

SOME FACTORS AFFECTING THE
STABILITY OF SYNAPSES IN
THE ADULT RAT

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ABSTRACT

(1) The synapses on rat hypoglossal neurones have been studied by light microscopy; in most cases, a Zinc Iodide-Osmium method was used to stain synaptic boutons. After one hypoglossal nerve has been injured, the number of synaptic boutons on the parent nerve cell bodies falls. It rises again after hypoglossal axons have reformed functioning neuro-muscular junctions.

(2) Dividing the ipsilateral lingual nerve at the time of axotomy impedes the restoration of bouton number to regenerating neurones.

(3) An electrophysiological study of the reflex responses of normal and transplanted hypoglossal nerves indicates that the synapses which form on neurones whose axons have regenerated to an unusual muscle are of similar origin to those present on the neurones before axotomy.

(4) Hypoglossal neurones loose boutons when silicone rubber cuffs containing colchicine or vinblastine are applied to their axons. These drugs do not impair effective nerve-muscle contact. Cuffs containing lignocaine partially block nerve-muscle conduction, but do not induce significant bouton loss.

(5) Retrograde axonal transport of horseradish peroxidase in the hypoglossal nerve was studied under a variety of conditions. Silicone rubber cuffs containing colchicine

or vinblastine inhibit retrograde transport. Those containing lignocaine do not.

(6) Bathing the divided hypoglossal axons in extracts of muscle did not prevent bouton loss.

(7) The possible role of axonal transport in maintaining central synapses is discussed.

(8) It is concluded that the motor axon terminal influences the synapses received by the nerve cell body. The restoration of boutons is also influenced by sensory information.

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INTRODUCTION

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Synaptic Structure

Localised dilatations of axons ending on the dendrites and somata of nerve cells were described by Held in the late nineteenth century. These end-feet, or boutons, were thought at first to be sites of cytoplasmic continuity between axons and neurones, but both Auerbach and Cajal reported fine lines of discontinuity between boutons and the neurone surface (1). The term "synapse" was introduced by Sherrington (2) to describe this coming together of axon and neurone at specialised junctions. But, although it was known that nerve impulses were delayed for some milliseconds as they passed across synapses (3-5), the absence of direct cytoplasmic continuity between presynaptic and postsynaptic elements was not confirmed until the introduction of electron microscopy (6-7).

The cytoplasm of a synaptic bouton is separated from that of the postsynaptic cell by a cleft about 20 nm across, bounded on either side by double membranes (6-9). The bouton often fits into a trough on the postsynaptic cell (6-7), and is surrounded by glial processes (7; 10), which also cover areas of neurone surface which are not involved in synapses (10). The cytoplasm of synaptic boutons contains mitochondria (6-8), and vesicles which vary in size and shape depending on the type of bouton (6-11) (see Experimental Section 2).

Vesicles

Most synaptic vesicles have a wall consisting of a single layer of membrane (8). Vesicles with clear centres range from 30 - 60 nm in diameter (8; 11-13) and may be either spherical or ellipsoidal, in aldehyde-fixed preparations (9-11; 13). There is some electrophysiological evidence that synapses associated with spherical vesicles are excitatory, while those with ellipsoidal vesicles are inhibitory (9-11). Boutons containing ellipsoidal vesicles take up glycine and gamma-amino-butyric acid - known to be inhibitory transmitters (17; 182; 207) - with high affinity (14-15); but boutons with spherical vesicles may do this too (16). Nevertheless, boutons with ellipsoidal vesicles seem more likely to take up inhibitory transmitters than terminals with spherical vesicles (17).

Axon endings which release catecholamines have larger vesicles (30-90 nm in diameter) with electron-dense cores (8; 12-13; 18-20). The proportion of vesicles with dense cores increases if tissue is incubated in noradrenaline (18-19), and decreases when stored amines are released by reserpine (8; 12; 21-22). Since some vesicles can retain their dense cores after losing their amine content (12; 18), the cores may not be pure transmitter.

Large dense-core vesicles (60 - 100 nm in diameter) are occasionally seen in the terminals of both cholinergic and adrenergic nerves (8; 12; 18); some contain catecholamines, but retain their dense cores after amines have been released with reserpine (12; 18).

There is considerable evidence in support of the

hypothesis that vesicles release transmitter substances into the synaptic cleft when nerve terminals are depolarised. Transmitter substances are released when nerve fibres are stimulated (4-5; 23-25). During the first few minutes of continuous nerve stimulation, the number of vesicles in axon terminals increases (26-27). Prolonged stimulation causes either no change or a slight depletion of vesicles, under normal circumstances (13; 26-28); but, if choline uptake is blocked with hemicholinium, stimulation induces a marked fall in vesicle numbers (27-28). This could mean that vesicle formation is linked to transmitter synthesis or that choline is directly required for making vesicles.

Structures indistinguishable from synaptic vesicles take up extracellular material into nerve terminals by pinocytosis (29-32); the rate of uptake is increased by nerve stimulation (29-30). Freeze-etched presynaptic membranes contain vesicle-sized pits, which increase in number during nerve activity (35-36).

Differential ultracentrifugation of brain homogenates yields a synaptosome fraction, comprising nerve terminals containing vesicles (8; 13; 33). Osmotic rupture of such synaptosomes releases a variety of transmitter substances (8; 13; 33).

Vesicles are thus associated with the storage and release of transmitter. Studies at the neuromuscular junction suggest that acetyl choline is released in quanta of uniform size (34); it seems probable that the release of acetyl choline from vesicles accounts for this.

The origins of synaptic vesicles are unclear.

Dense-core vesicles containing noradrenaline accumulate proximal to ligations of sympathetic nerves (22); while both clear and dense-core vesicles are seen in the proximal tip of a severed axon (37). The latter vesicles have been supposed to be formed locally from axonal microtubules (13; 37); but this seems unlikely because microtubules do not possess membrane components (12). In proximal axons, vesicles are seen in close relation to microtubules (38-40); it seems likely that some vesicles are formed proximally, and are transported down the axon along microtubules (see below).

The synaptic cleft is bounded by thickenings of both presynaptic and postsynaptic membranes (6-9), and sometimes contains filaments (8-9; 13). Most synapses in the mammalian central nervous system do not involve tight junctions between presynaptic and postsynaptic membranes (6-7; 9); but they must be firmly joined, since synaptosomes often have a fragment of postsynaptic membrane adherent (33). Collagen filaments may be involved in this linkage, since collagenase treatment causes nerve terminals to separate from muscle end-plates (41). (See Experimental Sections 2 and 3).

Interactions between nerve and muscle

A muscle with a normal motor nerve supply is usually only responsive to acetyl choline applied on or near to the end-plate region (42-44); it does not form functional contacts with implanted nerves (42; 45-46); and has acetyl cholinesterase activity at the end-plate (42-43;

If the motor nerve is cut, the area of muscle responsive to acetylcholine spreads out from the end-plate region (42-44; 47); so that, by two weeks, the whole fibre is as sensitive as the original end-plate (42). This is due to a twenty-fold increase in the number of receptor sites (42-43; 49). Denervation also permits implanted nerves to innervate the muscle at any site (42-43; 45-46; 48); and causes a decline in acetylcholinesterase activity (42; 46-47). (See Experimental Section 3).

Chronic electrical stimulation of denervated muscles partially prevents supersensitivity to acetylcholine (43-44; 50); but direct treatment of the muscle with acetylcholine does not (47). It is puzzling why fibrillation potentials, arising spontaneously in denervated muscles, do not have a protective effect similar to electrical stimulation. Concentrations of local anaesthetic agents which block the passage of action potentials down motor nerves do not induce supersensitivity to acetylcholine at muscle (51). Higher concentrations do cause supersensitivity, but this is probably due to blockage of axonal transport (44). Botulinus toxin prevents the release of acetylcholine, and induces muscle supersensitivity (42-43; 47); but it could be preventing the release of other factors too.

The relationship between muscle electrical activity and denervation supersensitivity is thus incomplete. If colchicine or vinblastine is applied to a motor nerve,

action potentials continue to pass and neuromuscular activity is maintained. The muscle, however, becomes supersensitive to acetyl choline (52). Low concentrations of colchicine and vinblastine, and high concentrations of local anaesthetics inhibit axonal transport (see below). It is possible that many of the changes associated with muscle denervation are due to the loss of materials, other than acetyl choline, which probably pass from nerve to muscle. This is supported by the fact that muscle supersensitivity appears sooner when the motor nerve is cut distally, than when it is cut proximally (42-43). Action potentials would cease to pass to the distal segments at the same time in both cases; but a longer distal nerve stump would continue to transport material to muscle for a greater time.

Nerves which do not form end-plates can prevent muscle denervation changes: adrenergic nerves stop muscle fibrillation after denervation (43), without forming synapses; but this could be due to a direct effect of noradrenaline on the excitability of muscle. Cultured muscles rapidly lose their cholinesterase activity unless sensory ganglia or homogenates of spinal cord are added to the medium (47). Similarly, the atrophy of denervated adult mammalian muscle in culture is prevented by foetal sympathetic ganglia (53). Acetyl choline and its analogues do not prevent cultured muscle from losing cholinesterase; but adenosine and cyclic adenosine monophosphate do (47).

There is evidence that the electrophoretic pattern

of muscle proteins changes if a normally "slow" muscle is innervated by a "fast" muscle nerve (54). The contraction characteristics of the muscle also change towards those of "fast" muscle (55); but these effects may depend on the pattern of impulses in the motor nerve.(42).

There is thus much evidence to suggest that motor nerves control the structure and function of muscle by transference of materials other than neurotransmitters. Proteins are released in direct proportion to acetylcholine when motor nerves are stimulated (56). Some proteins synthesised in neuronal perikarya are transported down motor nerves and enter muscle; while other neural proteins do not pass the neuromuscular junction (57). Possibly, some of these proteins control the expression of genes in muscle. Nucleotides, known to be released from stimulated nerves (58), might also control the activity of muscle enzyme systems directly (58-60).

Interactions between neurones

Trans-synaptic degeneration

Postsynaptic neurones show morphological changes if their presynaptic elements are injured. Such anterograde trans-synaptic degeneration can be observed in the relevant neurones of the lateral geniculate body after optic nerve section: 3 months after one eye has been removed, contralateral lateral geniculate cells of adult cats are smaller and have reduced nuclear and nucleolar size (61). Closing one eye of a newborn kitten has a similar effect (62). Comparable changes occur in the relay neurones of the auditory and olfactory systems, if their primary

afferent fibres are injured.(61). Structural changes also occur in neurones more than one synapse distant from a lesion: if one of a rabbit's eyes is removed at birth, after one month, the dendrites of neurones in the corresponding visual cortex have fewer spines than control cells (63). The visual cortex neurones of mice reared in darkness show similar changes (64). Likewise, second order relay neurones in the auditory and olfactory systems become smaller after lesions of their primary afferent fibres (61).

Changes can also be found in presynaptic neurones after damage to postsynaptic elements (retrograde trans-synaptic degeneration): lesions in the visual cortex cause reductions in size of both lateral geniculate and retinal ganglion cells (61). Comparable changes occur in the pyramidal cells of the human precentral gyrus after limb amputation (61).

There is thus evidence that damage to or a decrease in the activity of one part of a closed chain of neurones causes structural changes in the other members of the chain. The mechanisms responsible for these trans-synaptic changes are open to speculation. Nerve terminals are known to release proteins (56-57) and nucleotides (58) in addition to neurotransmitters; and extracellular material is taken into vesicles in synaptic boutons and is transported to their perikarya (39). In sympathetic ganglia, preganglionic nerve stimulation increases the synthesis of ribonucleic acid (65) and enzymes (60) in the ganglion cells; both effects are blocked by ganglion-

blocking drugs (60;65), and could be solely due to transmitter release. Adenosine is released when pieces of cerebral cortex are stimulated (58). Extracellular adenosine is known to increase the intracellular concentration of cyclic adenosine monophosphate (58); the latter can activate a protein kinase directly (59), so changing neuronal properties. The interdependence of synaptically linked neurones might depend on similar interactions.

There is some evidence that substances are transferred between neurones at synapses. If radio-active amino acids are injected into a mouse's eye, a small amount of the activity appears in the visual, but not the frontal, cortex (66). As no axons pass directly from retina to cortex, the transfer of material between the neurones of the visual chain may be presumed to go on across synapses. Such material could be regulating the metabolism of the neurones concerned. The nuclei of motor neurones bind increased amounts of Actinomycin-D after their axons loose contact with muscle (67); this might mean that a factor from muscle is regulating the expression of neuronal nucleic acids. Similar processes might be responsible for the above trans-synaptic effects. (See Experimental Sections 3-6).

Induced Changes in synaptic number

The most striking results have come from young animals subjected to lesions in early life. During the first few months of postnatal life, the number of synapses on rat cerebral cortex neurones increases linearly with

time (68). Both malnutrition (69) and hypothyroidism (70) impede this synaptic development. The lateral geniculate cells of rats reared in darkness have fewer synapses than controls reared in light (68). Similarly, if both the optic nerves of newborn kittens are crushed, the number of synapses on visual cortex neurones after six weeks is half that of controls; suturing both eyelids has the same effect (68). However, if the optic tracts of adult cats are cut, bouton loss at the visual cortex is much less, and takes place more slowly (71). (See Experimental Section 4).

When a dorsal root of an adult cat has been cut, the number of synapses on motor neurones in that region of the spinal cord decreases initially, but begins to rise again after 16 weeks. By 5 months the motor neurones have normal numbers of synapses (72). The restoration of boutons occurs without regeneration of the dorsal root fibres, and is probably due to collateral sprouts from nearby axons. Raisman has provided good evidence for such a mechanism: a lesion of the fimbrial afferents to rat medial septal neurones induces fibres from the medial forebrain bundle to form synapses on the vacated areas of septal neurones (73). Hemisection of the spinal cords of adult rats produces a more complicated series of structural changes (74): Motor neurones close to the lesion suffer significant losses of synaptic boutons during the first 20 days; but between the 20th and 30th days there is significant restoration of boutons. This is probably due to collateral sprouting by intact axons.

However, during the next 30 days, the motor neurones again loose boutons; by the 90th day they still lack 30% of their normal number of synapses. It may be that the motor neurones eventually reject some of the new boutons formed from collateral sprouts.

Whether a neurone forms a synapse with a particular axon depends on both pre-synaptic and post-synaptic factors. The evidence presented above (68;71) suggests that the activity of pre-synaptic elements is important, particularly during early synaptic development. But the state of the post-synaptic cell is also crucial. During the development of cerebellar synapses, Purkinje cells remain free of boutons for long periods, despite being surrounded by the terminals of parallel fibres (75). The persistence of post-synaptic membrane specialisations after pre-synaptic fibres have degenerated (76), and the presence of sub-synaptic cisterns before synapses form (75;77) suggest that localised changes in the neurone's membrane may dictate synaptogenesis. It is possible that transmitter receptor sites are directly involved in the acceptance of synaptic contacts: if areas of a frog's optic tectum are treated with α -bungaro-toxin (a venom which binds to acetyl choline receptors), regenerating optic nerve fibres do not synapse with the treated cells, but form connections with inappropriate parts of the tectum (78). The factors which determine whether a synaptic bouton is accepted or rejected are unknown. The interchange of messenger substances across the synapse, as proposed above for the

nerve-muscle relationship, might be involved. (See Experimental Sections 3-6).

There is evidence that changes in synaptic number can be induced by events more subtle than experimental lesions. Rats reared in an environment which has facilities for play and exploration develop larger cortical neurones, but have fewer synapses on each cell than rats kept in a restricted habitat (79). There have also been reports that synapses are removed in developing and adult mammals. During the first few weeks of post-natal life, some of the boutons on the spinal motor neurones of kittens become displaced by glial processes (80); while degenerating axon terminals, characterised by their contents of cisternae and concentric membranous bodies, have been seen in normal adult rats (81). Removal of synapses from the neurones of adult animals might thus continue throughout life.

Movement in the Nervous System

Cells and neurites in culture

Fibroblasts (82-86) and glial cells (87-88) advance over solid substances with gliding movements. The broad leading edge of the cell adheres to the substratum, and the tapered trailing edge is pulled in (88-89). The leading lamella of such motile cells shows characteristic "ruffling" activity (82-85; 87-88). "Ruffles" are lines of increased optical density observed at the cell surface, and are due to vertical extensions of sheets of cytoplasm (82-83). These extensions, or lamellaepodia, persist for usually less than a minute; so that the leading edge of

the cell undergoes constant localised protrusions and withdrawals.

Cultured nerve cells do not move, but they extend axons (neurites) into the surrounding medium. The most distal part of a neurite, the growth cone, shows both "ruffling" and "microspike" activity (88;90). The latter is more common, and is characterised by extension and retraction of long, thin, tapering cylinders (about 0.1-0.2 μm in diameter; 1-50 μm in length) from the growth cone (90; 208). Unlike motile cells, neurites continue to move in semi-fluid media (88); the formation of adhesions with the substratum does not seem essential for axon elongation. If proximal parts of growing axons are marked with carmine particles, they remain at a fairly constant distance from the cell body (91). This suggests that extensions of the growth cone are due to the addition of new cytoplasm and membrane materials to the distal end of the neurite, rather than to movements of the whole axon. (See Experimental Section 8).

Some of the structures within neurites and motile cells are comparable. Much of the length of axons contain longitudinally oriented microtubules (20-25 nm in diameter) and filaments (8-10 nm in diameter); the latter form regularly spaced arrays around microtubules (38; 40; 92-93). Glia and Fibroblasts also contain microtubules and filaments oriented along their long axes (85; 94-95). "Microspikes" extending from growth cones, and lamellaepodia at the "ruffled" membrane of motile cells do not contain these long structures;

instead, a meshwork lattice of short microfilaments (4-7 nm in diameter) is present (85; 87-88; 90; 92-93; 96).

If motile cells are treated with colchicine or vinblastine - drugs which disrupt microtubules - gliding movements stop, but the leading lamella continues to "ruffle" (87; 95; 97). The same drugs cause rapid cessation of neurite elongation, but growth cones continue to extend "microspikes" for some time after treatment (98). Microtubules are thus important for gliding movements of whole cells and for axon growth; they do not seem to play a part in "microspike" movements at growth cones or "ruffling" of leading lamellae.

The rapid linear movements of some intracellular organelles stop, and other organelles become abnormally distributed when the microtubules of cultured cells are disrupted with colchicine or vinblastine (97; 99-102). Intact microtubules are essential for axonal transport (see below); their role in axon growth may be to supply new materials to the growth cone (See Experimental Section 8).

Colchicine and vinblastine do not affect the microfilaments in "microspikes" or lamellaepodia (87; 95; 97). The microfilaments in motile cells form arrow-head complexes with heavy meromyosin (88; 103), and are therefore similar to muscle actin. There is less good evidence that microfilaments in neurite growth cones behave in the same way (88; 103). The drug cytochalasin-B changes the fine structure of the lattice microfilaments

in "ruffling" membranes and growth cones, and stops the extension of lamellaepodia and "microspikes" (87; 93; 96; 98). The movement of cells, growth of neurites, and axonal transport are all inhibited by this drug (87; 93; 98; 104). Cytocholasin-B does not affect the structure of microtubules and larger neurofilaments (87; 96)+ but it causes cultured cells to become multinucleate by inhibiting the formation of contractile bands of microfilaments (96).

The microfilaments of leading lamellae and growth cone "microspikes" have properties similar to actin, but are immunologically distinct from it (88; 103). The roles of the microfilaments appear to be : (a) extending and retracting lamellaepodia or "microspikes"; (b) collaborating with microtubules in axonal transport.

Axonal Transport

For more than fifty years, particles within axons have been known to make rapid longitudinal movements in both directions (105-106). Synaptic vesicles accumulate in the region immediately proximal to a ligature on a nerve (22; 37; 107); and are probably transported from the perikaryon down to the axon terminal. Proteins, synthesised from labelled amino acids in neuronal perikarya, pass down axons in separate fractions, and at different rates. The fastest moving of these is associated with small particles, and moves at a rate of about 410 mm/day (107-108); while the slowest moving material travels at about 2 mm/day (109). Intermediate rates of 208; 150; 40; and 6-12 mm/day have also been described (109; 113).

Extracellular protein is readily taken up into vesicle-like structures within nerve terminals (29-32); these are transported to the neurone perikaryon at rates varying from 34 to 280 mm/day (39;108; 110-112; 114). Most of the material which passes proximally is associated with membranes (114); either in vesicles or larger sacs and multivesicular bodies (39).

Axonal transport in either direction requires energy, and stops if neurones are deprived of oxygen (108) or exposed to metabolic inhibitors (108;115). Calcium ions may be needed to link material to the transport system, since anterograde transport of protein stops if neuronal perikarya are deprived of calcium; and labelled calcium is transported down axons at a speed similar to the fast rate for protein (116).

The drugs colchicine and vinblastine inhibit axonal transport in both directions (39; 107-109; 115; 117-118) (See Experimental Sections 5 and 7). Movement of intracellular organelles also ceases when cultured cells are treated with these drugs (99-102). Both drugs prevent cell division by stopping the formation of mitotic spindles (119). Cells and axons treated with colchicine or vinblastine show less of microtubules and increased numbers of filaments 8-10 nm in diameter (95; 97; 99-102; 107; 109; 118-121). This has caused some workers to suggest that normal neurofilaments and microtubules are interconvertible structures (122); but, since they have different amino acid compositions (123-124), this seems unlikely. One possibility is that the drugs alter the

configuration of microtubular protein from a tubular to a fibrillary form.

Since microtubules are disrupted by drugs which inhibit axonal transport, they have an implied role in the transport system. Cells bind colchicine in amounts roughly proportional to their content of microtubules (125); those with mitotic spindles, or motile cilia show the highest affinity. The molecule which binds colchicine is a dimer of the protein tubulin (molecular weight 60,000 daltons) (124;126). Microtubules have a diameter of 20-25 nm; their walls are 4-6 nm thick; and they have a clear central core 15 nm in diameter (92; 94). The wall of a microtubule has been described to consist of six filaments (92); while very high magnification electron micrographs suggest that it may be comprised of twelve circular subunits (127). Filaments of 8-10 nm diameter run parallel to microtubules, surrounding them in regular arrays (94); they appear to be made up of two coiled strands, each 3 nm in diameter, separated by a 3 nm central core (92; 123). Some workers have described thin filamentous links between microtubules and neurofilaments (94), and microtubules and synaptic vesicles (39;40). Near axon terminals, synaptic vesicles form regular rosettes around microtubules (38-40).

These inter-relationships have provoked suggestions that microtubules and neurofilaments co-operate in the axonal transport of membrane-bound material (108); but crayfish axons have no neurofilaments, yet exhibit axon transport (127).

Tubulin prepared from cilia migrates on electrophoresis identically with muscle actin, and has an amino-acid composition similar to actin (128). The colchicine-binding protein from brain interacts with muscle myosin, increasing ATP-ase activity and viscosity in a way similar to actin (129). But spindle tubules and microtubules in neurones do not bind heavy meromyosin (103); and antibodies to actin do not react with tubulin (124). Thus if microtubules do have actin-like activity, this is not due to tubulin.

Microtubules in the outer fibre doublets of cilia have ATP-ase activity; and this is largely due to a protein fraction, separate from tubulin, which forms the short arms of the microtubules (130-132). This protein, dynein, can be further fractionated into α -dynein (370, -380,000 daltons) and β -dynein (355, -360,000 daltons)+ α -dynein has most of the ATP-ase. Intact microtubules seem needed to achieve the full ATP-ase activity of brain α -dynein (131-132). The fine side arms observed on microtubules in axons (39-40; 94) may be dynein.

The presence of a protein with Ca^{++} and Mg^{++} stimulated ATP-ase associated with microtubules strengthens the evidence in support of the hypothesis that microtubules play a key rôle in axonal transport. The reliance of transport on Ca^{++} (116) adds further weight.

Nerve terminals contain neurostenin, an acto-myosin-like contractile protein with two components: neurin-like actin; and stenin-like myosin (133-134). Stenin has ATP-ase activity and contractile properties when combined

with neurin; vesicle fractions of brain homogenates contain stenin, while the membrane portions of synaptosomes have neurin (133-134). The stenin of vesicles may therefore interact with the contractile proteins of microtubules in providing the motile force for axonal transport; and with neurin in nerve terminals to produce release of vesicle contents.

One can only speculate on the functions of axonal transport. It probably supplies structural components (135), enzymes (114) and transmitter substances (107) to nerve terminals. Some proteins transported in axons pass to post-synaptic sites (57; 66); and proteins labelled in muscles can be traced up motor axons (136). The possible "trophic" roles of such substances have already been discussed.

EXPERIMENTAL SECTION

1. GENERAL METHODS

(a) Animals

Male albino rats, aged 3 months at the time of first operation and between 3 and 10 months at time of death, were studied. There were four rats in a cage (cage size: 38 cm long; 25 cm wide; 18 cm deep); they had free access to standard food pellets and water. The animal room was maintained at 22-23°C; lighting was entirely artificial, providing alternating 12 hour periods of light and dark.

(b) Preparation of tissue

Rats were anaesthetised either with ether or by intraperitoneal injection of 25% urethane (0.7 ml/100 g. body weight). Urethane was used when electrophysiological investigations were carried out before death. Anaesthetised rats were killed by exsanguination, with or without perfusion of the vascular system with fixative. Specimens of brain and peripheral nerves were promptly removed and processed for histology.

(c) Microscopy and Photography

Histological material was examined using a Vickers M-41 Photoplan light microscope. Quantitative analysis was carried out at X400 magnification, using either conventional transmitted light or phase contrast. Photomicrographs were taken with the M-41 camera and coupled photometer. Photographs of operations and dissections were taken using a Zeiss binocular dissecting microscope with a camera attachment. Kodak Plus -X Pan film was used throughout, and was developed in Kodak D 19 developer and fixed in Kodafix.

(d) Electrophysiology

Electromyographic responses were measured using a specially made electrode comprising two fine silver wires, insulated except at their tips, which had been passed down the barrel of a 25 gauge hypodermic needle and cemented in place at the tip with Aradite. Potentials were displayed on an Electrophysiological Instruments Limited C.E.P.T.U. system which comprised a double-beam oscilloscope, a.c. preamplifier, and a coupled square pulse stimulator. Nerve stimulation was achieved by passing pulses from the above unit through a 1:1 isolation transformer to a pair of silver hook electrodes. Potentials were measured direct from the oscilloscope; some single sweeps were photographed with a Cossor oscilloscope camera.

(e) Analysis of data

Raw data from microscopic or electrophysiological measurements was subjected to statistical analysis using an Olivetti Programma 101 calculator. Significance was detected by reference to Documenta Geigy (7th Edition).

(f) Materials (except common chemicals)

Colchicine ; BDH Chemicals Limited Poole, Dorset, England.

Horseradish peroxidase ; BDH Chemicals Limited.

OCT Compound ; Ames Company, Miles Laboratories, Stokepoges, Slough, England.

Silastic Medical Adhesive. Silicone type A. (Dow Corning, U.S.A.) Lepetit Pharmaceuticals, Maidenhead, England.

Vinblastine Sulphate ; Eli Lilly & Co., Ltd., Basingstoke, England.

2. METHODS FOR COUNTING SYNAPTIC BOUTONS

(a) ZINC IODIDE-OSMIUM (Z10)

Rats were killed by exsanguination and the muscles overlying the occiput were removed to expose the occipital bone, atlas and atlanto-occipital membrane. The arch of the atlas was removed and the occipital bone nibbled away to reveal the medulla oblongata, fourth ventricle and cerebellum. The latter was pushed rostrally and a transverse cut made across the medulla about 2 mm rostral to the obex. A second transverse cut was made 2-3 mm caudal to the obex, and the piece of medulla between the two cuts removed (FIG. 1). The left hand side of the medulla was marked by slicing off a small piece from its lateral extremity (FIG. 2). The tissue was then transferred to a Petri dish containing artificial cerebrospinal fluid (CSF), chilled to 4°C and of the following composition :

Na Cl , 121.5 mM ; NaHCO₃, 25 mM ;
KCl, 3.5 mM ; CaCl₂ , 1.3 mM ;
MgCl₂ , 1.14 mM ; NaH₂PO₄ , 0.51 mM ;
Urea, 3.33 mM ; glucose, 3.33 mM.

The dish containing the specimen and CSF was taken to a refrigerated room at 4°C and left for 2-3 minutes. The medulla was placed on the plate of a McIlwain tissue chopper, which had been pretreated with 0.5 ml of OCT compound, to stop tissue from being lifted by the chopper blade. Transverse slices 0.4 mm thick were cut at right angles to the long axis of the medulla. The sliced

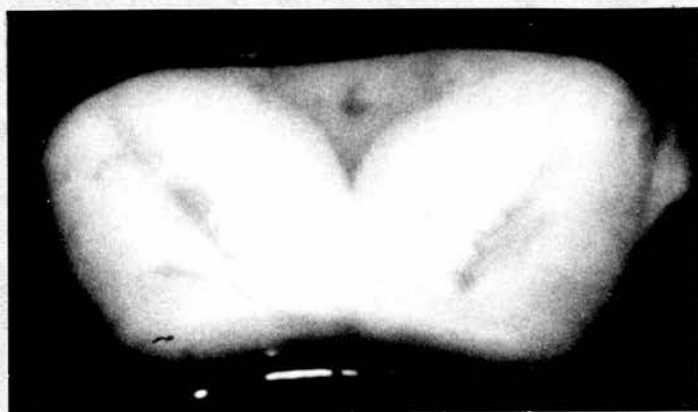


FIG. 1. Piece of rat medulla oblongata containing the hypoglossal nuclei. The darker inverted triangle is the caudal half of the floor of the fourth ventricle; the central apex of this is the obex.



FIG. 2. As above, but the left side has been marked by slicing off a small piece from its lateral extremity.

medulla was replaced in chilled CSF and the individual slices were separated with watchmaker's forceps.

Usually about 6 slices could be obtained from the area 1 mm rostral to 1 mm caudal to the obex; these were dropped into 2 ml of freshly prepared Z10 mixture at 4°C, and left at this temperature for 18-24 hours.

Z10 mixture was prepared as described by Akert and Sandri (137) :

- (1) Dissolve 5 g. crystalline iodine in 200 ml distilled water.
- (2) Slowly add 15 g. pure zinc powder, stirring constantly, Zinc iodide forms during an exothermic reaction.
- (3) Stir the mixture overnight.
- (4) Filter and store the resultant zinc iodide solution at 4°C.
- (5) To make 2 ml Z10 reagent, add 0.6 ml 2% aqueous osmium tetroxide to 1.4 ml of the stock zinc iodide solution. The mixture turns a straw yellow colour.

After overnight impregnation in Z10, medulla slices were treated as follows:

- (1) Wash in three 15 ml changes of 0.2 M phosphate buffer pH 7.4.
- (2) Dehydrate in graded aqueous ethanol solutions; 15 minutes in each of 50%; 70%; 90%; 95% ethanols; then two periods of 10 minutes in absolute alcohol.
- (3) Clear in two changes of benzene; 15 minutes in each.
- (4) Impregnate for 3 periods of 30 minutes in changes of paraffin wax/plastic mixture at 60°C. (Paraplast;

melting point 56-57°C.)

- (5) Embed each slice in a separate plastic mould filled with wax. Usually four blocks were prepared from each animal.
- (6) Cut 3 μ m sections on a hand rotary microtome.
- (7) Float sections out on water at 50°C.
- (8) Mount on gelatine coated glass slides and dry at 37°C overnight.
- (9) Remove wax by soaking in two 15 minute changes of xylene.
- (10) Mount in DPX under glass coverslips
- (11) Dry over a warm hot-plate for 3-6 hours before microscopy.

Microscopy

(1) Finding the hypoglossal nuclei

The hypoglossal nuclei are discrete collections of large neurones (about 50 μ m in diameter) lying in the posterior part of the medulla, on either side of the deepest part of the fourth ventricle (FIG. 3) (138). In their most rostral parts, the nuclei lie close to the floor of the fourth ventricle and are separated by about 0.5 mm (FIG.4). The most caudal parts of the nuclei lie deeper, immediately adjacent to the central canal (FIG. 5). Usually, sections taken from midway between these extremes were used for quantitative microscopy.

(2) Appearance of Z10 stained tissue

The Z10 stained tissue was seen to be studded with



FIG. 3. Low power view of a section cut transversely through rat medulla at the level of the obex. The hypoglossal neurones (XII) lie on either side of the fourth ventricle (IV). Staining was by Z10.

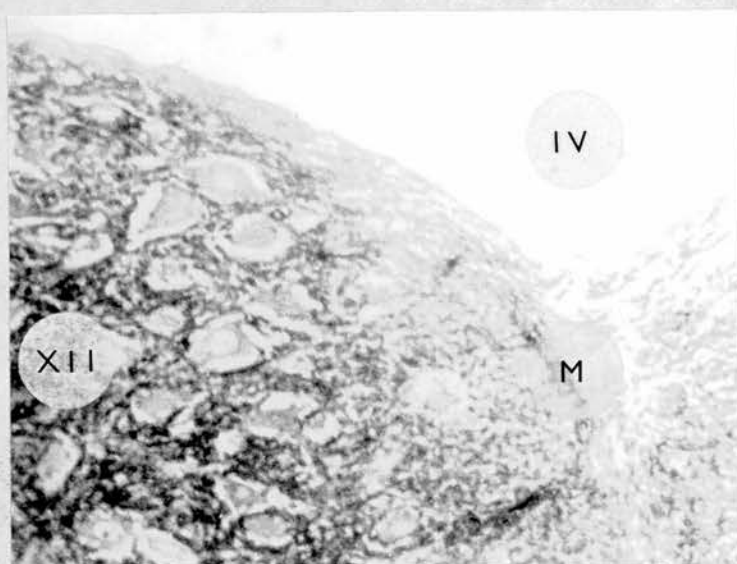


FIG. 4. Section at level of the most rostral hypoglossal neurones (X11); they lie close to the floor of the fourth ventricle (1V), about 0.5 mm from the mid-line (M).

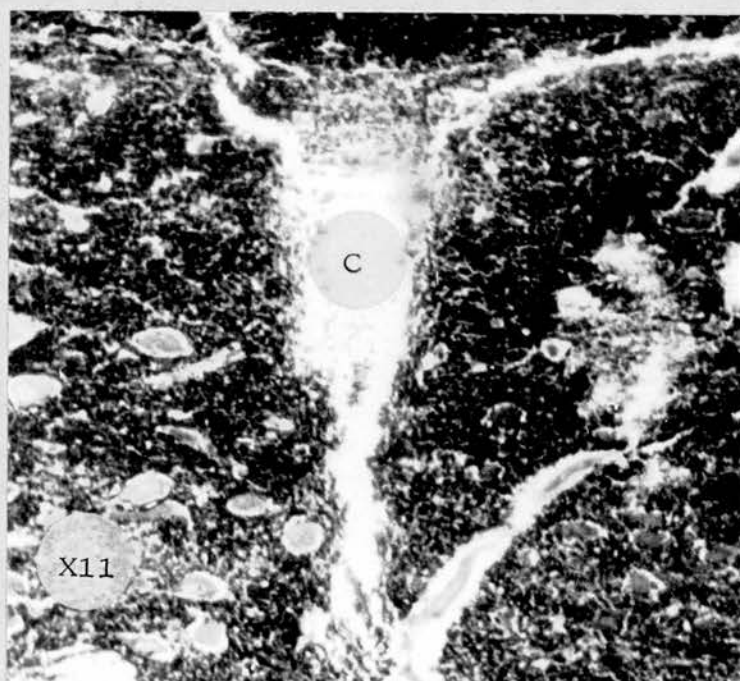


FIG. 5. Section at level of the most caudal hypoglossal neurones (X11); they lie on either side of the central canal (C).

black granules, distributed mainly in the neuropil and surrounding nerve cell bodies (FIGS. 6-8). Where large dendrites were identifiable, they were also closely surrounded by densely stained granules. Neuronal perikarya showed a variable amount of less dense particulate matter within the cytoplasm. The nerve cell body surface often shrank away from the surrounding neuropil; but many boutons remained adherent to the neurone.

(3) Procedure for counting synaptic boutons

Shrinkage of neuronal perikarya from the neuropil permitted the surface of individual neurones to be identified. The number of synaptic boutons remaining in contact with each neurone perimeter could therefore be counted; counts were registered on a small hand tally. Where the plane of section caused the neurone perimeter to be continuous with part of a large dendrite, the dendrite boutons were included in the count; however, in most cases, counts were of somatic boutons only. For one rat, the boutons on one hundred neuronal profiles were counted; fifty profiles were selected randomly from each hypoglossal nucleus. To minimise errors due to differences in stain penetration, identical numbers of neurones were studied from both left and right hypoglossal nuclei in any one section.

From the bouton counts, calculations of mean boutons per neurone perimeter; standard deviations and errors; and the significance of differences between groups were made. Student's 't' test was employed for the comparison of groups of neurones.

FIG. 6.

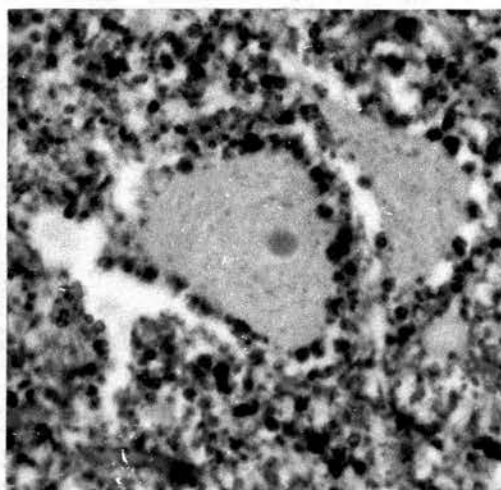


FIG. 7.

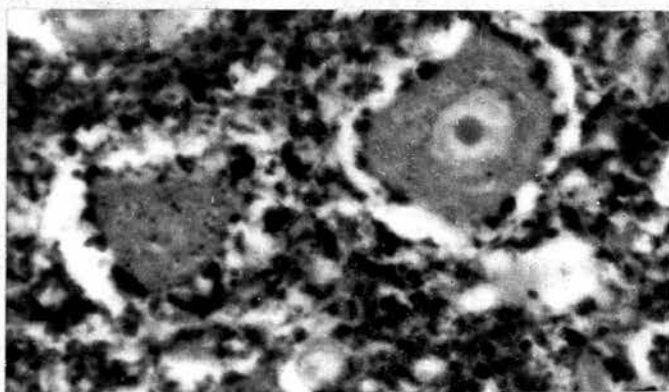
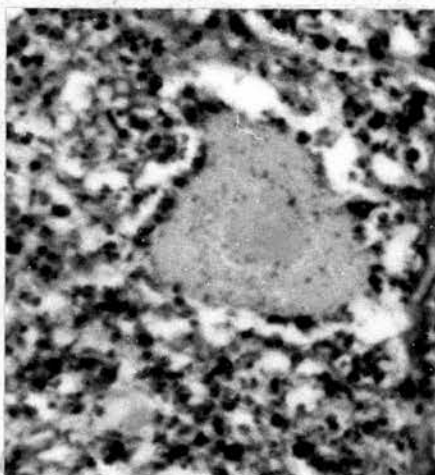


FIG. 8.



FIGS. 6-8. High power photomicrographs of normal hypoglossal neurones stained with Z10. Note that the perikarya shrink away from the neuropil. The surface of the cells is studded with black granules which are also present in the neuropil; these are synaptic boutons.

DISCUSSION

Champy introduced iodides of osmium as histological stains in 1913 (139). Maillet (140) found that nerve fibres and their terminals were well stained by a mixture of zinc iodide and osmium tetroxide; staining was not due to the presence of catecholamines, since it was unaffected by reserpine administration (141). Maillet suggested that lipo-protein was the substrate for Z10 staining (141). Electron microscopic evaluations of the Z10 method have shown that synaptic vesicles at known cholinergic sites stain densely, while dense-core vesicles do not (137;142). Z10 was thought to be specific for cholinergic boutons (143), but its staining of both spherical and ellipsoidal vesicles is against this (144).

The Z10 substrate and reaction product remain unknown; the staining of ellipsoidal vesicles (144) and persistence of staining after hemicholinium (142) make it unlikely that acetyl choline is the substrate. Lipo-proteins in vesicle and other membranes seem more likely sites of action (141; 143).

The dense granules in the neuropil and on neuronal surfaces described above are thus synaptic boutons. The paler cytoplasmic staining is probably due to mitochondria, lysosomes, golgi apparatus and smooth endoplasmic reticulum which have been reported to be stained in other work using Z10 (143; 145). Restricting counting to dense granules at the neurone perimeter will have excluded much of this cytoplasmic material from the counts.

Electron micrographs of Z10 stained tissue from the present study confirm staining of synaptic vesicles and mitochondria in boutons (see Appendix 1. Electron micrographs were kindly prepared by Miss C.H.M. Ivens).

Shrinkage of the neuronal surface from the neuropil makes it easier to identify the perimeters of individual nerve cells, but it may have pulled off some somatic boutons. The pre-synaptic and post-synaptic elements in synaptosome preparations are often firmly bound together (33); this is probably what keeps many boutons adherent to the perikarya of the neurones shown in FIGS. 6-8. A decrease in the adhesiveness of boutons may therefore be seen as a loss of boutons from the shrunken perikarya. This possibility is discussed further below.

2 (b) MODIFIED BIELSCHOWSKY SILVER METHOD

Rats were anaesthetised with ether and fixed by perfusion through the left cardiac ventricle with 40 ml 10% formal-saline after a pre-wash of 20 ml 0.9% sodium chloride containing 0.04% sodium nitrite as a vasodilator. Medullae were excised as described in 2 (a), and left in 10% formalin for 2 days. The staining procedure was basically that described by Armstrong et al. (146), but the slight modification suggested by Sumner and Grant (147) was used.

- (1) Transfer fixed medullae to an aqueous solution containing:
5% potassium dichromate
2% chromium fluoride

Leave in this for 5 days.

- (2) Leave in 5% aqueous potassium dichromate at 37°C for 2 weeks.
- (3) Wash in running water for 24 hours.
- (4) Transfer to molten 25% aqueous gelatine at 37°C. Leave 24 hours.
- (5) Transfer to fresh gelatine and keep at 4°C for 1 hour to harden.
- (6) Cut out blocks of gelatine containing medullae and keep in formal-calcium until sectioned.
- (7) Wash in water for 2 hours.
- (8) Cut 10 μ m sections on a freezing microtome and collect them in distilled water.
- (9) Transfer sections to polythene tea strainers.
- (10) Place in 5% ammonium hydroxide at 52°C for 30 Minutes.

- (11) Rinse in 3 changes of distilled water, 30 minutes each; and leave in distilled water at room temperature overnight.
- (12) Impregnate in 10% aqueous silver nitrate at 37°C for 15 minutes.
- (13) Rinse in distilled water.
- (14) Take through 4 changes of 20% neutral formalin at 37°C for a total of 10 minutes.
- (15) Rinse in distilled water.
- (16) Transfer to an evaporating dish containing silver diamine solution, prepared from :
30 ml 10% aqueous silver nitrate with enough strong ammonium hydroxide to redissolve the precipitate, followed by 15 drops of excess ammonium hydroxide and 3 drops of pyridine. Warm and agitate until sections turn golden brown.
- (17) Transfer to 1% ammonium hydroxide for 1 minute.
- (18) Then to 1% acetic acid for 1 minute.
- (19) Tone in warmed 0.2% gold chloride for 3 minutes.
- (20) Rinse in distilled water
- (21) Reduce in 2% aqueous oxalic acid for 3 minutes.
- (22) Rinse in distilled water.
- (23) Transfer to 5% aqueous sodium thiosulphate for 5 minutes.
- (24) Rinse in distilled water.
- (25) Transfer to a dish containing :

80% ethanol)	
)	in equal parts
1.5% aqueous gelatine)	

Leave for 5 minutes.

(26) Mount on glass microscope slides; blot with tissue paper; harden gelatine in 95% ethanol for 5 minutes.

(27) Dehydrate in absolute alcohol.

(28) Clear in xylene.

(29) Mount in DPX under glass coverslips.

Microscopy

The hypoglossal nuclei were identified as in 2 (a). Dense black dots were prominent in the neuropil and surrounding neuronal perikarya. As with Z10 (2 (a)), neurones had often shrunken away from the neuropil, but many dots remained adherent to their surfaces. The cytoplasm of hypoglossal perikarya showed variable degrees of particulate staining; but this was always paler than the dense perineuronal dots (FIGS. 9-10). Boutons were counted around neurone perimeters, exactly as in 2 (a).

DISCUSSION

Brain and spinal cord tissue stained in blocks with Bielschowsky's original silver nitrate/pyridine method show staining of neurones and variable impregnation of synaptic boutons (148-149). The boutons often contain dense ring-shaped structures, and electron microscopy has shown that this is due to deposition of silver grains onto neurofilaments (149). The amount of filamentous material varies from bouton to bouton, and therefore staining of individual terminals is unreliable (149).

Immersion of the fixed tissue block in potassium

FIG. 9.

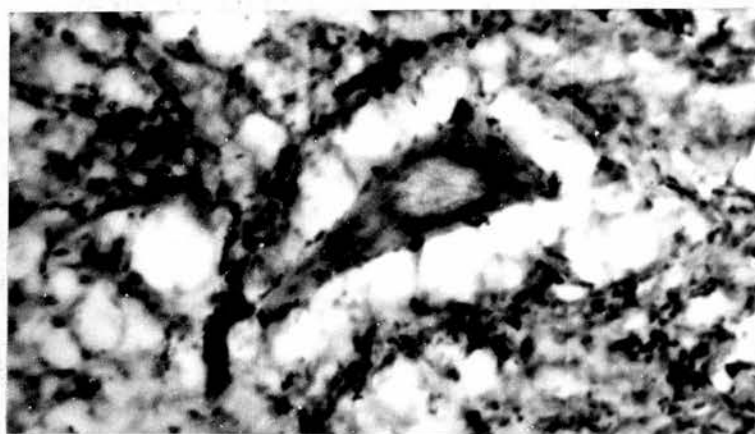
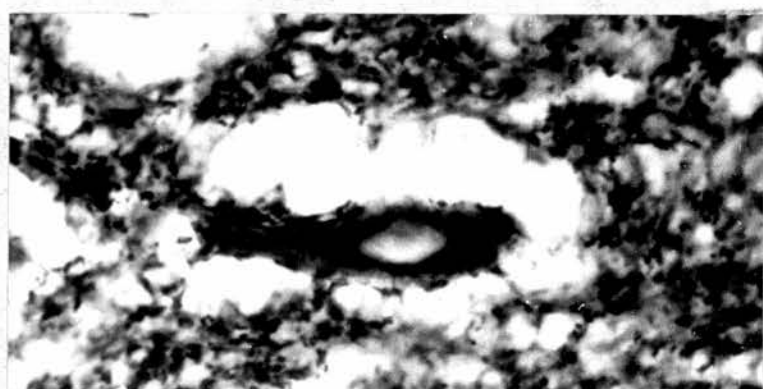


FIG. 10.



FIGS. 9 & 10. High power photomicrographs of normal hypoglossal neurones stained by the modified Bielschowsky method. Note that the perikarya shrink away from the neuropil. The black granules at the neuronal surface and in the neuropil are synaptic boutons.

dichromate solution before sectioning and silver impregnation ("post-chroming") preserves lipids and phospholipids, and gives much more consistent staining of all types of bouton (146; 150-151). Although mitochondria are stained in perikarya and boutons (146; 150-151), the most densely staining structures are synaptic vesicles (147). This modified Bielschowsky method stains all types of bouton, irrespective of vesicle types; the lipid content of vesicle envelopes may be the substrate rather than vesicle contents (147).

The black dots described in the neuropil and at neuronal perimeters thus represent synaptic boutons. The problems of neuronal shrinkage and bouton adhesion are the same as discussed in 2 (a). Z10 and modified Bielschowsky methods give similar results; neuronal shrinkage is more severe with the latter. The two methods differ in the time needed to prepare specimens for microscopy: the silver method takes 3 to 4 weeks, while Z10 takes only 3 to 4 days.

3. EFFECTS OF HYPOGLOSSAL NERVE INJURY AND TRANSPLANTATION

(a) USING Z10

METHODS

Five groups of rats were observed over a period of 14 months. Operations were carried out under light ether anaesthesia using sterile instruments and a "no touch" technique.

Group 1 contained 17 rats. The left hypoglossal nerve was divided as it passed deep to the posterior belly of the digastric muscle (FIGS. 11 and 12).

Group 2 contained 13 rats. The left hypoglossal nerve was crushed at the same site as Group 1.

Group 3 contained 10 rats. The left hypoglossal nerve was divided as above, and its central end implanted into the left sternomastoid muscle. This was achieved by transfixing the nerve with a fine silk suture; tying a loose knot; pulling the nerve through the muscle, and leaving a small knot of silk to hold the nerve in place. (FIGS. 11-15).

Group 4 contained 6 rats. They were treated as in Group 3, then, 123 days later, the left spinal accessory nerve was divided as it entered the deep aspect of the superior end of the sternomastoid muscle.

Group 5 contained 2 rats. These were normal animals.

Between 3 and 120 days after operation, rats were killed and their medullae prepared for microscopy by the Z10 method described in 2 (a). Boutons on neurones

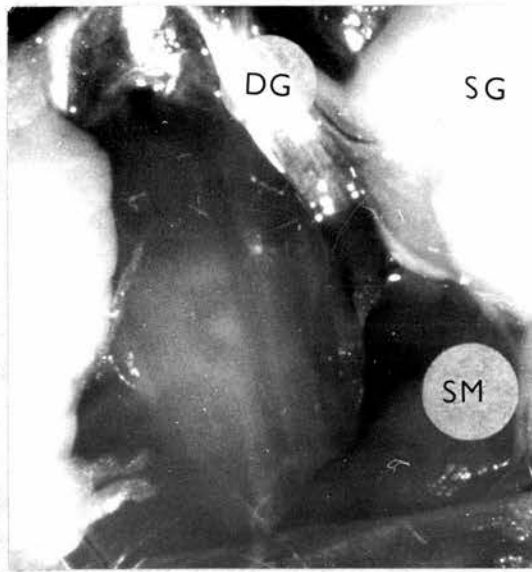


FIG. 11. Preliminary dissection of neck of rat, showing posterior belly of digastric muscle (DG); sternomastoid muscle (SM); and retracted submandibular gland (SG).



FIG. 12. The posterior belly of the digastric muscle has been retracted to expose the left hypoglossal nerve.



FIG. 13. The hypoglossal nerve is transfixed with a fine silk suture.

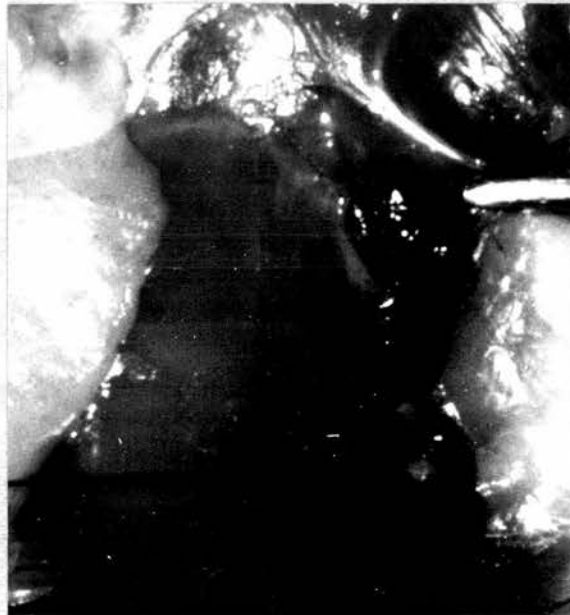


FIG. 14. The central stump of the divided nerve is pulled through the sternomastoid muscle.



FIG. 15. The implanted nerve is held in place by a loose knot of silk.

in both hypoglossal nuclei were counted and compared using student's 't' test.

RESULTS

Results of bouton counts are shown in TABLE 1 and expressed graphically in FIG. 16. In the latter, mean boutons per neurone perimeter in the left hypoglossal nucleus are expressed as a percentage of those on the right, and plotted against days after operation on a log time scale.

Normal animals (Group 5) showed no significant difference between left and right hypoglossal nuclei. After division of the left hypoglossal nerve (Group 1), the number of boutons per neurone perimeter fell on the axotomised side, and remained low between the 10th and 30th days after axotomy. Restoration of bouton number then followed, and was almost complete by the 60th day.

After crushing the hypoglossal nerve (Group 2), the number of boutons decreased in the same manner, but returned to normal earlier. After transplanting the hypoglossal nerve into sternomastoid (Group 3), the number of boutons continued to fall, and by 120 days only 40% remained. Division of the left spinal accessory nerve at this stage (Group 4) was followed by a slow return of boutons.

Photomicrographs of axotomised neurones are shown in FIGS. 17-19 (cf. normal neurones in FIGS. 6-8); they show loss of somatic boutons.

TABLE 1

Group	Days after operation	Mean boutons per neurone perimeter			p
		Left	Right	L/R x 100	
1	3	45.3	53.5	84.7	<0.0005
	7	22.4	36.2	61.9	<0.0005
	9	21.36	28.79	74.2	<0.0005
	14	28.16	46.41	60.7	<0.0005
	16	12.96	24.37	53.2	<0.0005
	21	18.16	30.59	59.4	<0.0005
	24	28.6	45.9	62.3	<0.0005
	27	20.7	34.8	59.5	<0.0005
	28	27.6	45.94	60.1	<0.0005
	34	31.3	41.5	75.4	<0.0005
	35	27.98	37.98	73.7	<0.0005
	41	49.3	60.2	81.9	<0.0005
	42	36.56	43.82	83.4	<0.0005
	48	54.9	62.9	87.3	<0.0005
	49	22.84	28.62	79.8	<0.0005
	59	39.51	43.64	90.5	<0.05; >0.025
	66	39.1	47.22	82.8	<0.0005
2	7	35.4	55.5	64.8	<0.0005
	7	17.34	25.24	68.6	<0.0005
	9	45.97	65.70	71.0	<0.0005
	14	22.96	29.78	77.1	<0.0005
	16	54.9	76.4	71.8	<0.0005
	21	16.4	20.2	81.2	<0.0005
	27	51.35	60.51	84.9	<0.0005
	28	37.86	45.58	83.1	<0.0025; >0.0005
	35	39.82	46.94	84.8	<0.0005
	42	53.52	49.8	107.5	<0.1; >0.05
	52	54.26	57.46	94.4	<0.1; >0.05
	59	37.76	42.68	88.5	<0.005; >0.0025
	66	37.28	35.5	105.0	<0.2; >0.15
3	7	26.02	32.48	80.1	<0.0005
	14	15.98	33.34	47.9	<0.0005
	21	12.1	19.76	61.2	<0.0005
	28	25.64	49.58	51.7	<0.0005
	35	32.04	56.37	56.8	<0.0005
	42	34.04	62.42	54.5	<0.0005
	52	27.82	42.76	65.1	<0.0005
	59	26.76	52.98	50.5	<0.0005
	66	35.52	62.66	56.7	<0.0005
	120	21.22	50.96	41.6	<0.0005
4	10	28.68	56.68	50.6	<0.0005
	17	21.0	36.88	56.9	<0.0005
	31	30.36	47.42	64.0	<0.0005
	45	28.76	39.1	73.6	<0.0005
	69	23.7	33.06	71.7	<0.0005
	83	29.12	33.06	88.1	<0.01; >0.005

TABLE 1 (continued)

Group	Days after opera- tion	Mean boutons per neurone perimeter			p
		Left	Right	L/R x 100	
5	0	57.1	58.2	98.1	>0.1
	0	37.04	34.26	108.1	<0.05 >0.025

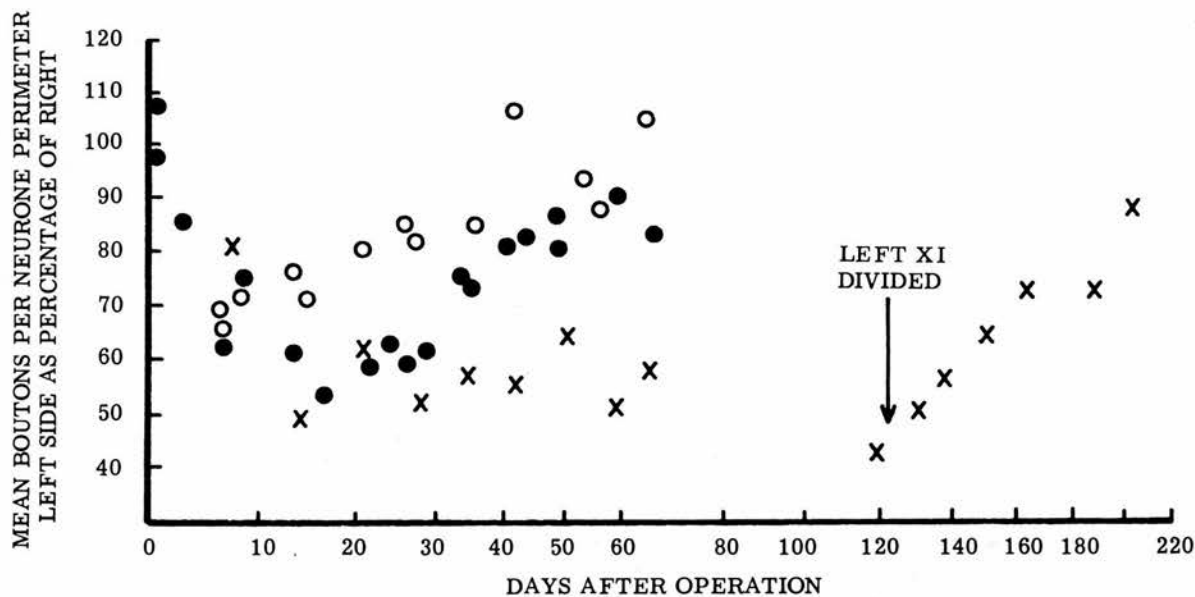


FIG. 16. Mean boutons/neurone perimeter in the left hypoglossal nucleus are expressed as a percentage of those on the right, and plotted against days after operation (Log. time scale). Filled circles = Left hypoglossal nerve divided. Open circles = Left hypoglossal nerve crushed. X = Left hypoglossal nerve divided and implanted into sternomastoid muscle. Arrow indicates time of division of Left spinal accessory nerve. Each point represents one animal. All points below 90% on ordinate indicate highly significant differences between left and right nuclei ($P < 0.005$).

FIG. 17.

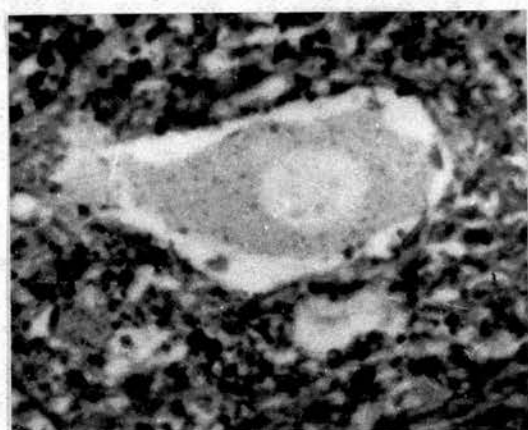
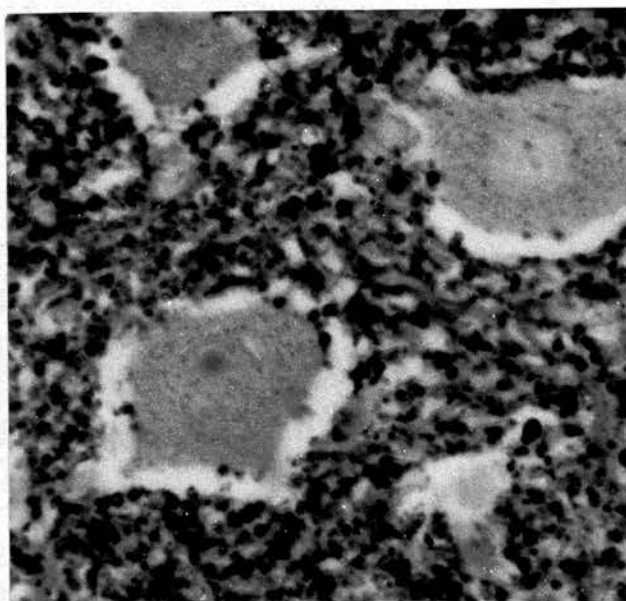


FIG. 18.

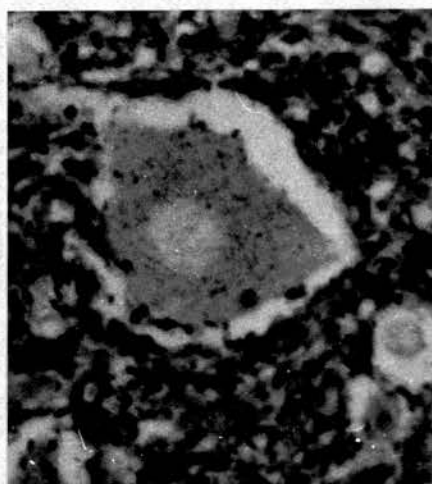


FIG. 19.

FIGS. 17-19. Hypoglossal neurones 14 days after division of hypoglossal nerve; stained by Z10. Note the marked reduction in numbers of somatic boutons. (cf. Figs. 6-8).

(b) USING THE MODIFIED BIELSCHOWSKY METHOD

METHODS

Observations were made upon 14 rats over a period of two months. Two rats were unoperated controls; the rest were killed at intervals after the left hypoglossal nerve had been crushed as it passed deep to the posterior belly of the digastric muscle. Histology was carried out as in 2 (b) and microscopic analysis performed as in 2 (a).

RESULTS

Results of bouton counts are shown in TABLE 2 and FIG. 20. In the latter, mean boutons per neurone perimeter in the left hypoglossal nucleus are expressed as a percentage of those on the right, and plotted against time after operation. Photomicrographs of axotomised neurones are shown in FIGS. 21 -- 22 (cf. normal neurones in FIGS. 9-10); they show loss of somatic boutons.

Control animals had no significant differences between left and right hypoglossal nuclei. Four days after left hypoglossal crush, there was a significant fall in bouton numbers on the axotomised side. This was greatest at 7 days, and, thereafter, restoration of bouton numbers occurred. By 56 days there was no significant difference between operated and control sides.

TABLE 2

Days after operation	Mean boutons per neurone perimeter			p
	Left	Right	L/R x 100	
0	41.22	42.32	97.4	<0.25; >0.20
0	39.58	43.30	91.4	<0.01; >0.005
4	29.16	43.88	66.5	<0.0005
4	30.46	42.92	70.9	<0.0005
7	23.62	38.00	62.2	<0.0005
7	25.66	39.94	64.2	<0.0005
13	28.72	41.44	69.3	<0.0005
13	23.18	35.28	65.7	<0.0005
24	22.10	27.50	80.4	<0.0005
24	22.02	31.68	69.5	<0.0005
42	24.76	30.62	80.9	<0.0005
42	28.50	29.08	98.0	<0.35; >0.30
56	32.60	33.08	98.5	<0.4; >0.35
56	24.35	25.51	95.5	<0.15; >0.10

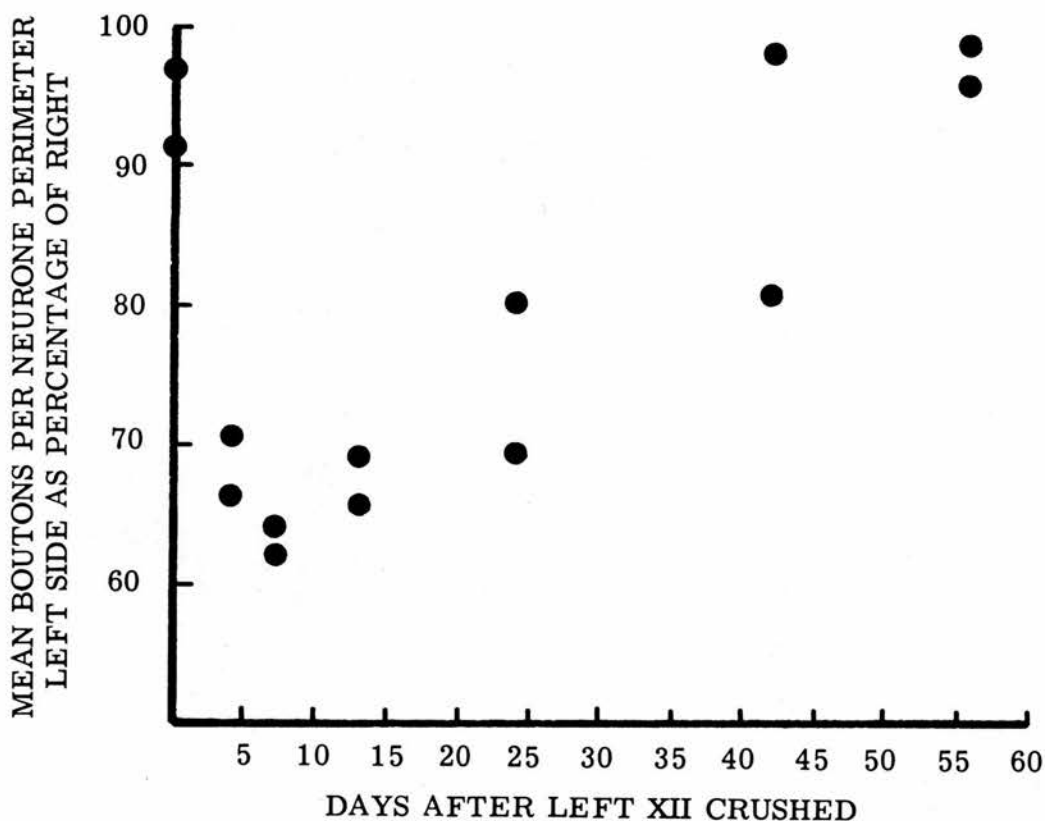


FIG. 20. Hypoglossal neurones - modified Bielschowsky stain. Mean boutons/neurone perimeter in the left hypoglossal nucleus are expressed as a percentage of those on the right, and plotted against time after left hypoglossal nerve crush.



FIG. 21.

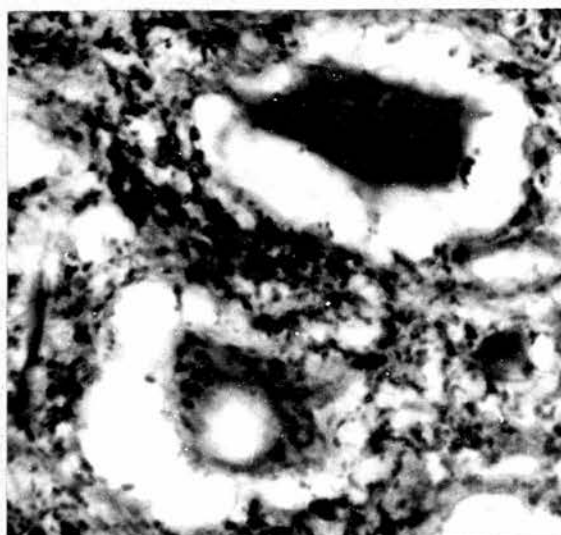
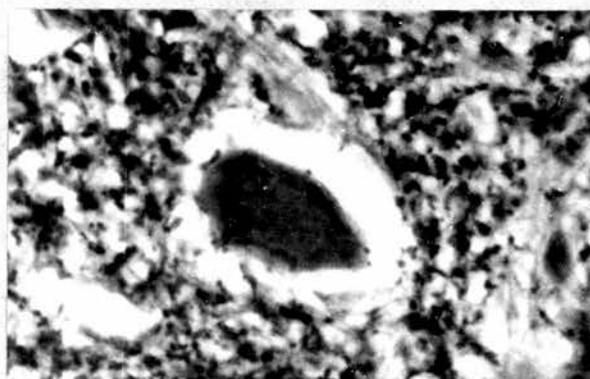


FIG. 22.



FIGS. 21 & 22. Hypoglossal neurones, stained by modified Bielschowsky method, 13 days after hypoglossal nerve crush. Note marked reduction in numbers of somatic boutons (cf. Figs. 9 & 10).

DISCUSSION OF 3 (a) & 3 (b) RESULTS

Axotomy induces a series of structural and metabolic changes in the parent nerve cell bodies and their surrounding glia. Swelling of the perikaryon, in the order of a 20% increase in cross-sectional area, begins soon after axotomy (152) and is greatest between 1 and 4 weeks; after which, cell size gradually returns to normal (153-154). Dispersion of ribosomal nucleic acid (Nissl substance) gives the neuronal cytoplasm a pale powdery appearance after axotomy; but this "chromatolysis" is not seen in all species (152;155). The nuclei of axotomised neurones increase in size, take up an eccentric position, and show increased nucleolar prominence (153). There is an increase in neuronal nucleic acid content which starts at the nucleolus (156) and is followed by an increased rate of protein synthesis and dry cell mass (157). Glycolytic enzymes increase in activity after axotomy, while oxidative processes (158) and synthesis of enzymes concerned with transmitter functions decrease (152).

Glial cells surrounding axotomised neurones show increased nucleic acid synthesis (159); but individual types of glia behave differently. Astrocytes undergo two increases in dry mass; first between 1 and 10 days after axotomy, and second when the axons reform functional neuromuscular contacts (160). Hypertrophy of myelinating oligodendroglia coincides with the second astrocytic response; and, like the latter, does not occur if the

axons are prevented from forming effective contact with muscle (160).

Dendrites retract after axotomy (161-162), and re-expand when effective nerve-muscle contact is restored (162). Such dendritic shifts might be expected to disturb relationships between synaptic boutons and the injured neurones. Axotomised motor neurones do show reduced monosynaptic excitatory responses (163-166); the changed shape of the post-synaptic potentials suggests selective failure of somatic rather than dendritic synapses (163). Inhibitory trans-synaptic responses have been reported to be reduced (163) or prolonged after axotomy (164). In sympathetic ganglia, post-ganglionic axotomy causes progressive failure of the post-ganglionic response to pre-ganglionic stimulation (167-170). This begins 1-3 days after axotomy (169-170) and is accompanied by persistence of a normal output of acetyl choline on pre-ganglionic stimulation (167). Since progressively larger amounts of acetyl choline need to be applied to discharge the axotomised ganglion cells (167-168), the number of post-synaptic receptor sites may be reduced.

Previous structural investigations on pre-synaptic terminals synapsing onto axotomised neurones have given conflicting results. Schadewald (171) failed to detect changes in bouton numbers after cranial nerve division in cats; but Barnard, using similar light microscopic methods, found that ventral root section in cats caused a reduction in the number of synaptic boutons on spinal motor neurones (172). But the latter work is difficult

to interpret, since dorsal roots were cut in some cases. Using electron microscopy, Blinzinger and Kreutzberg (173) reported a qualitative reduction in boutons on axotomised facial nerve neurones of rats. Glial cells were seen interposing between neuronal perikarya and pre-synaptic terminals. Scanning electron microscopy has shown that axotomised neurones have smoother surfaces than normal (174); but Barron et al. failed to show displacement of boutons from cat brachial motor neurones after brachial plexus section (175). This may have been due to concomitant sensory fibre damage causing boutons to swell or to fail to retract.

The work presented in 3 (a) and 3 (b) indicates that boutons are shed from the surface of axotomised rat hypoglossal neurones. Comparison of FIGS. 18 and 22 shows that Z10 and modified Bilschowsky methods yield very similar results. Electron microscopic studies, carried out concurrent with the present work, have confirmed that boutons are displaced and later restored to the injured hypoglossal neurones of rats (176). Tongues of glial cytoplasm have been reported to separate boutons from axotomised motor neurones (176) and sympathetic ganglion cells (170). Most of the displaced boutons contain spherical vesicles, while ellipsoidal vesicle boutons are little affected (177). Subsurface cisterns, which normally lie in post-synaptic cells beneath points of bouton contact, disappear after axotomy; but return before bouton numbers are restored (77). Synaptic sites thus appear to be determined by

post-synaptic factors.

Schadewald's failure to detect bouton loss with the light microscope (171) may have been due to lack of shrinkage in his preparations; since, when the neurone surface is closely opposed to the neuropil, it is difficult to tell which boutons are in contact. The shrinkage of neurones described here (2 (a) and (b)) may have enhanced the displacement of boutons after axotomy, both by rendering apparent boutons that had separated and by pulling off terminals less adherent than normal. Neuropil is less adherent to neurones following axotomy (178), and so some potentially displaced boutons may have been lost during shrinkage.

Although neuronal swelling (153-154) could cause an apparent reduction in bouton density, this will have been partially counteracted by counting boutons per neurone perimeter. Secondly, hypoglossal neurones whose axons have been implanted into sternomastoid are not swollen at 120 days, but the number of boutons is markedly reduced.

The present results indicate that bouton numbers return to normal sooner after nerve crush than after nerve division. Crushed nerves regenerate to muscle sooner than divided nerves (179), and so the trigger for bouton replacement may rely on the establishment of nerve-muscle contact. However, transplanting the divided hypoglossal nerve into normal sternomastoid muscle does not prevent bouton loss; instead bouton numbers continue to fall, and are only restored if the normal nerve

supply to sternomastoid is cut.

Nerve fibres implanted into normally innervated muscle fail to form synapses (45-46). However, if the normal nerve supply to the muscle is cut, implanted nerve fibres form functional end-plates within a few days (42; 45-46; 48; 180), both at sites of previous end-plates and at entirely new sites on the muscle (46; 48).

The factor initiating restoration of bouton numbers is thus directly related to the formation of effective nerve-muscle contact by the regenerating axons. After hypoglossal nerve crush, functional nerve-muscle contact is first detectable on the eighth day (see section 3 (a)); bouton numbers do not begin to rise until some 5-7 days later. It is interesting that the second of the two astrocytic responses to axotomy, the oligodendroglial response and the re-expansion of retracted dendrites all depend on the reformation of effective nerve-muscle contact (160; 162).

The nature of the signal for the bouton changes is investigated further in subsequent sections.

4. EFFECTS OF LINGUAL NERVE DIVISION

METHODS

Operations

Three groups of rats were operated under light ether anaesthesia using sterile instruments and a "no touch" technique.

Group A Contained 9 rats. The left lingual nerve was divided at the point where it crossed the submandibular duct.

Group B Contained 8 rats. The left lingual nerve was divided as above, but, in addition, the left hypoglossal nerve was crushed as it passed deep to the posterior belly of the digastric muscle.

Group C Contained 8 rats. The left hypoglossal nerve was crushed as above, but the lingual nerves were not injured.

Histology

Twenty-eight days after operation, animals were killed and slices of medulla were impregnated by Z10, as in 2 (a). Microscopy and counting were carried out as above. To exclude subjective bias, the identification code on each slide was covered with opaque tape, and a randomly allocated working code printed on the tape. Thus, the observer assessed each slide "blind". From each animal, 100 neuronal profiles were assessed; equal numbers coming from right and left hypoglossal nuclei.

Expression of results and statistics

Group A Bouton counts from 450 profiles (pooled data from 9 animals) in the left hypoglossal nucleus were compiled to give a mean value for boutons per neurone perimeter. The corresponding value for the right hypoglossal nucleus was calculated, and the two values compared by Student's 't' test.

Groups B and C Bouton counts from 400 profiles (pooled data from 8 animals) in the left hypoglossal nuclei of Group B rats were compared by Student's 't' test with those derived from the left hypoglossal nuclei of Group C rats. Similarly, the right hypoglossal nuclei of Group B rats were compared with those of Group C rats.

RESULTS

Comparisons of the various groups and sides are shown in FIG. 23. Standard errors of the means and the significance of the differences between each pair are shown.

Division of the left lingual nerve alone caused no significant change in hypoglossal perisomatic boutons (Group A; FIG. 23/I). The right hypoglossal neuronal boutons in Groups B and C did not differ significantly (FIG. 23/II). However, left lingual nerve division together with left hypoglossal crush (Group B) inhibited significantly the restoration of bouton number to the injured neurones when compared with hypoglossal crush alone (Group C), ($P = < 0.0005$; FIG. 23/III).

DISCUSSION

After one hypoglossal nerve has been crushed, the number of synaptic boutons on the parent neurone cell

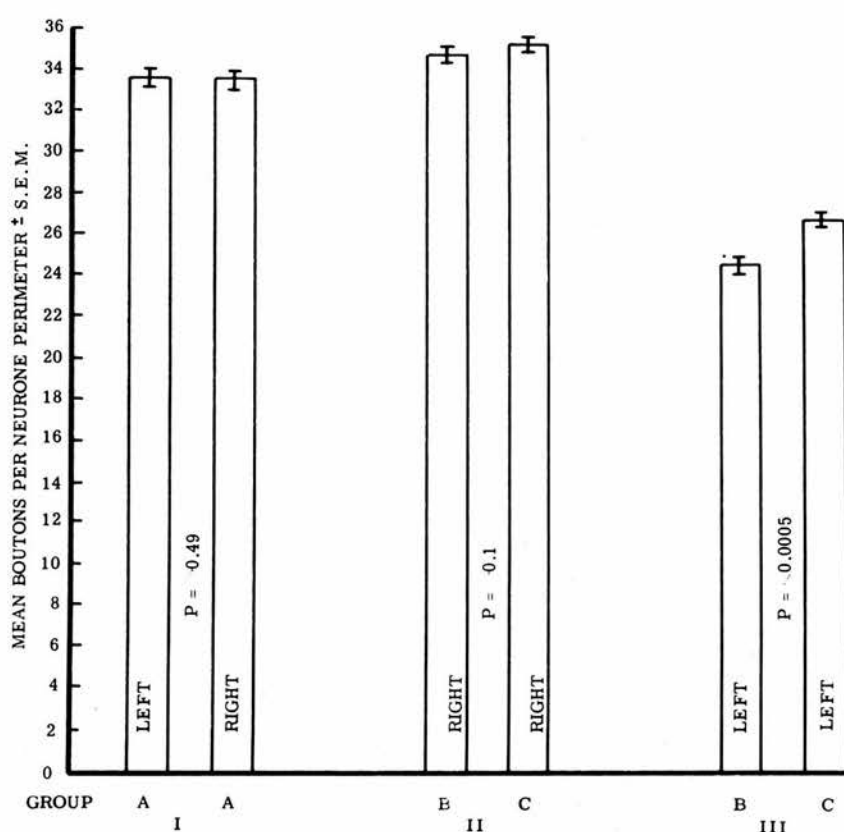


FIG. 23. Mean boutons/neurone perimeter, together with standard errors. Significance of difference between each pair of values calculated by Student's 't' test.

Group A = Left lingual nerve divided.

Group B = Left lingual nerve divided; left hypoglossal nerve crushed.

Group C = Left hypoglossal nerve crushed.

Note : Highly significant difference between left sides of Groups B and C ($P = < 0.0005$).

bodies decreases (see 3 (a) and (b)). This loss of boutons is greatest 7 days after axotomy; injured neurones then have about 65% the number of boutons of uninjured controls. After effective nerve-muscle contact has been restored, the number of boutons returns to normal; by 28 days after nerve crush, the cells have about 80% of their full complement of somatic boutons. The present study was undertaken to see if division of the ipsilateral lingual nerve would affect boutons on normal and regenerating hypoglossal neurones. The interval of 28 days was chosen because this is the period during which boutons are being most rapidly restored. (See FIG. 16).

Lingual afferents to hypoglossal neurones

Unilateral lingual nerve stimulation in the cat (181) and the rat (182) has both excitatory and inhibitory effects on hypoglossal neurones bilaterally. The ipsilateral excitatory response begins at 4-7 ms latency and is larger than the contralateral response which occurs some 1-2 ms later (183). A later, more variable, inhibitory effect frequently follows the initial excitatory response (182-183). It is probable that interneurones in the spinal nucleus of the trigeminal nerve relay this lingual-hypoglossal reflex (184). The lingual nerve thus affects hypoglossal neurones bilaterally, but only via interneurones.

Changes after lingual nerve division

The present study indicates that ipsilateral lingual nerve division causes no detectable loss of boutons on

normal hypoglossal neurones after 28 days. But during hypoglossal nerve regeneration, when synaptic contacts are reforming on the motor neurones, lingual nerve section significantly inhibits the restoration of bouton number. This could mean that synaptic connections undergoing change are more vulnerable to the effects of sensory nerve division than established contacts. Alternatively, the small loss of boutons caused by lingual nerve division could be more easily detected on axotomised cells, since total boutons are less.

The pre-synaptic boutons of afferent fibres degenerate if a lesion is made between the terminals and their ganglion cells (72;185). When boutons are separated from the lesion by one or more synapses, the situation is less clear. Crushing the optic nerves of newborn kittens causes a 50% reduction in synaptic bouton frequency at visual cortex cells after 6 weeks (68). This may depend on loss of afferent impulses, since eye closure has the same effect (68). In adult cats, however, optic tract section causes a much smaller loss of cortical boutons, which is not detectable until 2 months after operation (71).

In the present work, the boutons studied are separated from the divided lingual nerve by at least one synapse. Failure to detect bouton changes on normal hypoglossal neurones after lingual nerve division may have been due to the short period of study and the maturity of the animals. That lingual nerve division impairs the restoration of bouton number to regenerating neurones may mean that, as in the developing synapses of young animals,

nerve connections are more labile to changes in afferent stimulation during their formation than when they are established. Another possibility is that synaptic connections undergoing change are more susceptible to trans-synaptic degeneration due to loss of "trophic" factors from their primary afferent fibres.

5. EFFECTS OF SILASTIC CUFFS CONTAINING DRUGS

METHODS

Preparation of cuffs

Cuffs were made from room temperature vulcanising silicone rubber (Silastic RTV-A) as follows :

0.1% colchicine and 0.1% vinblastine cuffs were made by mixing 1 ml silastic adhesive with 1 mg colchicine or 1 mg vinblastine sulphate. 20% Lignocaine cuffs were made by mixing 1 ml silastic with 200mgs. Lignocaine hydrochloride. Pure silastic (control cuffs) or silastic-drug mixture was loaded into a 2 ml syringe and air was expelled. The contents were then pushed through a 25 gauge hypodermic needle (of which the tip had been broken off) onto Petri dishes coated with PTFE to aid release. Under a $\times 10$ binocular dissecting microscope, rings of silastic were squeezed out onto the dishes and allowed to vulcanise for 24-48 hours at room temperature. The resulting cuffs had an approximate internal diameter of 1 mm; an external diameter of 2 mm; and were about 0.5 mm thick (see FIG. 24). Approximately 100 cuffs were made from 1 ml silastic; each contained either no drug or approximately 10 μ g colchicine; 10 μ g vinblastine or 2 mg Lignocaine. Vulcanised cuffs were peeled off the dishes with watch-maker's forceps and dipped briefly in sterile saline before implantation. Dishes containing cuffs were kept at 4°C until use.

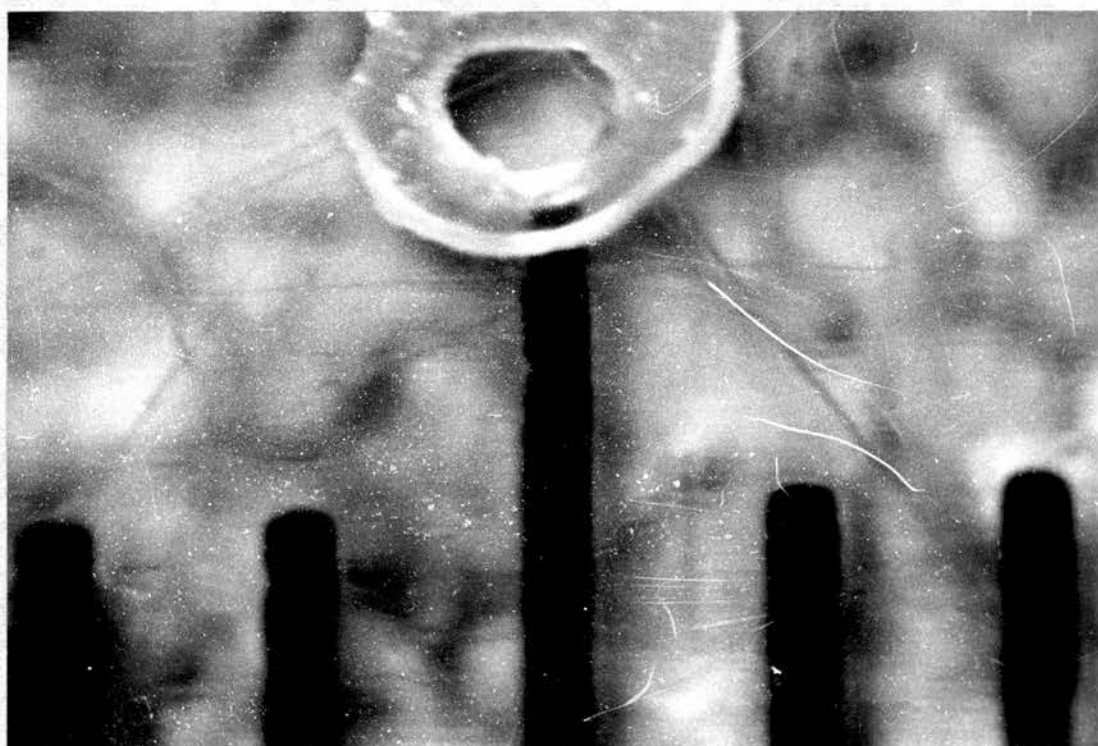


FIG. 24. Silastic cuff laid against a ruler marked in 1 mm graduations.

Operations

Four groups of rats were studied.

Group A contained 6 rats. Under light ether anaesthesia, the left hypoglossal nerve was exposed by retracting the posterior belly of the digastric muscle (FIG. 25).

Under a binocular dissecting microscope, a 0.1% colchicine cuff, whose circumference had been cut with fine scissors, was hooked around the nerve (FIG. 26); great care was taken not to damage the nerve. The digastric muscle was then replaced over the cuffed nerve. A control cuff containing no drug was similarly applied to the right hypoglossal nerve.

Group B contained 5 rats. 0.1% vinblastine cuffs were applied to the left hypoglossal nerves and control cuffs to the right, as above.

Group C contained 6 rats. 20% lignocaine cuffs were applied to the left hypoglossal nerves and control cuffs to the right, as above.

Group D contained 12 rats initially; 3 were excluded from analysis as explained below. At the initial operation, the left hypoglossal nerve was divided and transplanted into the ipsilateral sternomastoid muscle as in 3 (a). One rat died after operation. 9 weeks later the remaining 11 rats were re-operated by dividing the left spinal accessory nerve as in 3 (a). After 2 more weeks, the transplanted nerve was exposed and stimulated at 12 pulses/sec and using hook electrodes. In 9 of the 11 rats stimulation caused twitching of the left sternomastoid muscle; in the remaining 2 rats, the cut hypoglossal nerve



FIG. 25. Dissection of rat neck. The posterior belly of the digastric muscle has been retracted to expose the hypoglossal nerve.



FIG. 26. A silastic cuff has been slit at one point on its circumference, and hooked around the hypoglossal nerve.

had formed a neuroma and there was no response to stimulation. The latter rats were excluded from further involvement. The remaining 9 rats were divided into two groups: in one group (4 rats) a plain silastic cuff was placed around the left hypoglossal nerve; in the other (5 rats), a 0.1% colchicine cuff was placed around the left hypoglossal nerve.

Electrophysiology

Animals in Groups A, B and C were anaesthetised with urethane 14 days after implantation of the silastic cuffs. The hypoglossal nerves were exposed and stimulated proximal to the cuff with silver hook electrodes at 2-4/sec. The electromyographic response (EMG) was recorded from the corresponding side of the intrinsic tongue muscles with a bipolar electrode as described above. To standardise results, the electrode was always inserted near the tongue tip and in to one or other side to a depth of 1.5 cms. The voltage amplitude of the EMG evoked by a maximal stimulus was measured (see FIG. 27). Mean values for drug treated and control rats were compared by Student's 't' test.

Histology

Axons (Groups A,B and C)

After EMG measurements had been made, 1-1.5 cm segments of each hypoglossal nerve were removed so that each specimen included the cuffed region. Nerves were transferred to 10% formol-saline and then secondarily fixed in Susa (186) for 24 hours. The staining procedure used was basically that of Holmes (186) :

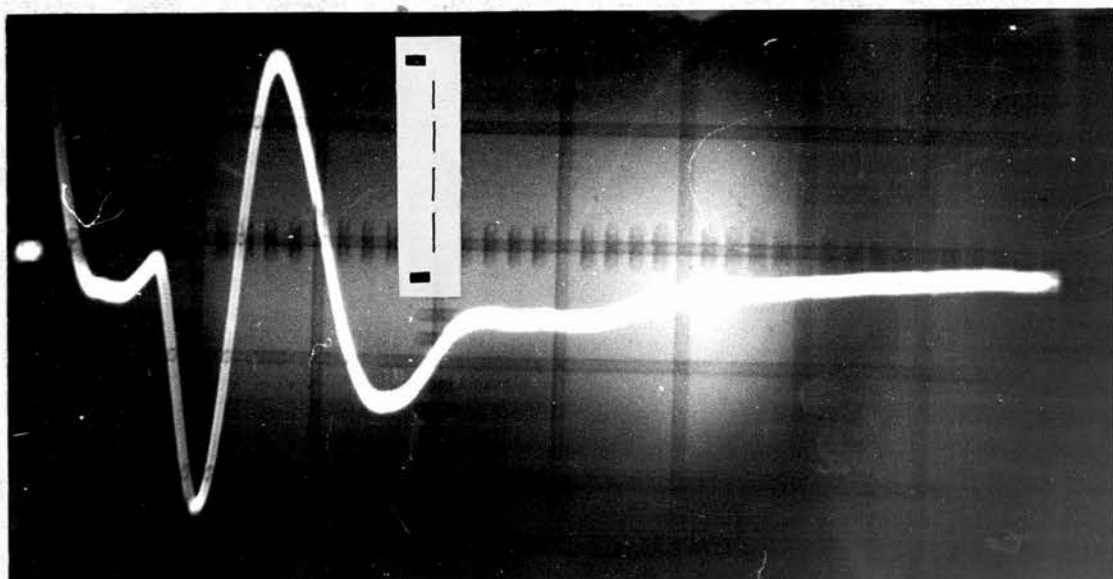


FIG. 27. Oscilloscope photograph of the EMG recorded from tongue intrinsic muscle of rat after a single square pulse was delivered to the hypoglossal nerve.

Stimulus - 18 v.

Time base - 2 ms/large division.

Sensitivity - 5 mv/ large division.

Inset thick lines indicate method used for measuring the size of EMG response.

- (1) Dehydrate fixed nerves in two 30 minute changes of 95% alcohol followed by two similar periods in absolute alcohol.
- (2) Clean in benzene; 30 minutes in each of two changes.
- (3) Impregnate in three 30 minute changes of paraffin wax in a vacuum oven at 60°C. Embed in plastic moulds filled with wax.
- (4) Cut 10 μ m longitudinal sections and mount on gelatine coated slides. Dry overnight at 37°C.
- (5) Remove wax for 15 minutes in xylene.
- (6) Transfer to Lugol's iodine solution for 5 minutes.
- (7) Transfer to 5% sodium thiosulphate solution for 5 minutes.
- (8) Wash slides in water for 10 minutes, and rinse in distilled water.
- (9) Transfer to 20% aqueous silver nitrate; leave in dark oven at 37°C for 2 hours.
- (10) Wash in three 10 minute changes of distilled water.
- (11) Impregnate in a solution containing:
 - 50 ml boric acid buffer
 - 45 ml borax buffer
 - 494 ml distilled water
 - 1 ml 1% aqueous silver nitrate
 - 5 ml 10% pyridineImpregnate overnight at 37°C.
- (12) Transfer to reducing solution containing :
 - 1 g. hydroquinone
 - 10.g. sodium sulphite
 - 100 ml distilled water

Leave for two minutes.

- (13) Wash in water for 3 minutes; rinse in distilled water.
- (14) Tone in 0.2% gold chloride for 3 minutes.
- (15) Rinse in distilled water
- (16) Transfer to 2% oxalic acid for 5 minutes or until axons appear blue-black
- (17) Rinse in distilled water
- (18) Fix in 5% sodium thiosulphate for 5 minutes
- (19) Wash in water for 10 minutes
- (20) Dehydrate in graded alcohols
- (21) Clean in xylene for 10-15 minutes
- (22) Mount in DPX under glass coverslips
- (23) Dry over warm hot plate for 3-6 hours.

Boutons

Groups A, B and C. After the hypoglossal nerves had been removed, rats were exsanguinated, and their medullae impregnated in Z10 as in 2 (a).

Group D. 22 days after silastic cuffs had been applied, rats were killed and their medullae impregnated in Z10 as in 2 (a).

Microscopical analysis

Axons. Silver stained hypoglossal nerve sections from Groups A, B and C were examined as follows: 10-15 sections of each hypoglossal nerve were examined at X400 under transmitted light. Using an eyepiece with a circular graticule, a fixed area was selected from the cuffed region of each nerve section. The cuffed region was usually identified by the slight indentation in the

nerve sheath caused by the cuff (FIG. 36). In each section, the number of axons crossing the graticule area were counted. The mean number of axons crossing the graticule in the cuffed area was calculated for each hypoglossal nerve; and the drug treated nerves were compared with controls by performing Student's 't' test on the pooled data.

Boutons

In Groups A, B and C, Z10 sections were analysed for boutons as in 2 (a). Mean boutons per neurone perimeter on drug treated sides were compared with controls by performing Student's 't' test on the pooled mean values.

In Group D, slides were assessed in a double coding procedure as in Section 4. Thus the observer assessed slides 'blind' to the type of cuff being used. Boutons were counted as before; for each animal the mean boutons per neurone perimeter on the left side were expressed as a percentage of those on the right. Pooled results from colchicine treated animals were compared with those from controls by student's 't' test.

RESULTS

Groups A, B and C

Boutons

Colchicine and Vinblastine containing cuffs caused significant reductions in boutons per hypoglossal neurone perimeter when compared with control cuffs. Lignocaine cuffs caused no significant bouton changes in the group as a whole; but two rats showed small significant

reductions in hypoglossal neurone boutons. Photomicrographs of affected neurones are shown in FIGS. 28-33. Numerical results are tabulated in TABLE 3 and pooled data is expressed in FIG. 34.

FIG. 28.

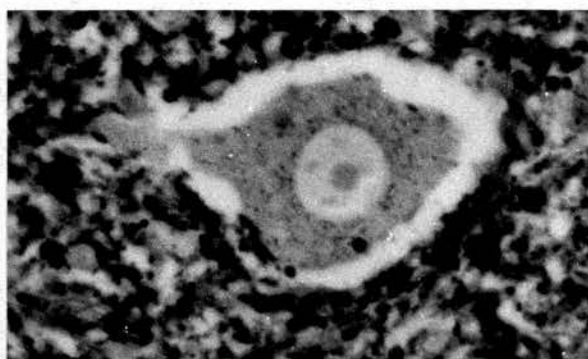


FIG. 29.

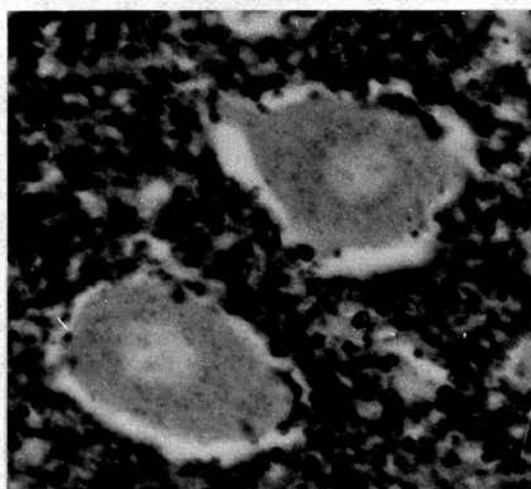
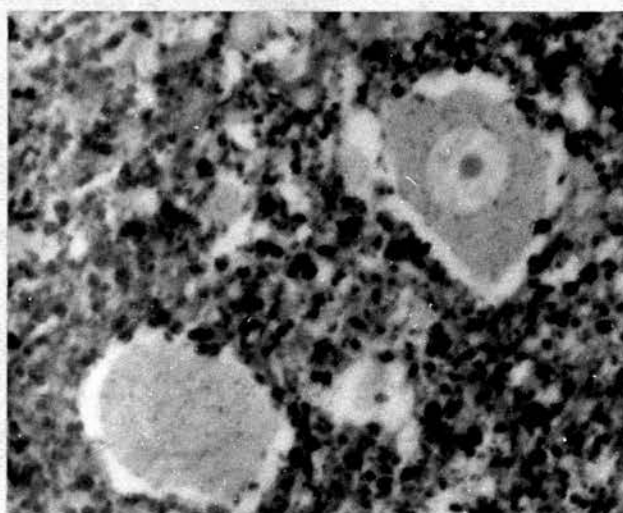


FIG. 30.



FIGS. 28-30. Hypoglossal neurones - Z10 stain - 14 days after silastic cuffs containing 0.1% colchicine had been applied to the hypoglossal nerve. Note : reduction in numbers of somatic boutons. (cf. Figs. 6-8).

FIG. 31.

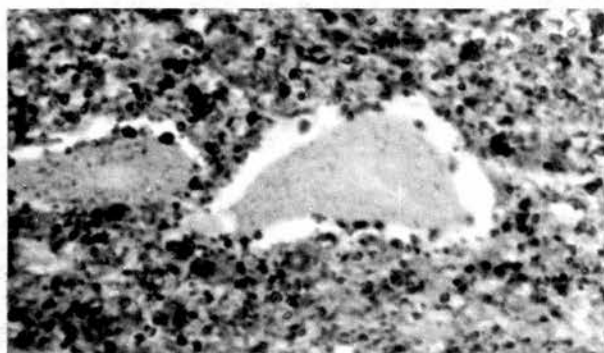


FIG. 32.

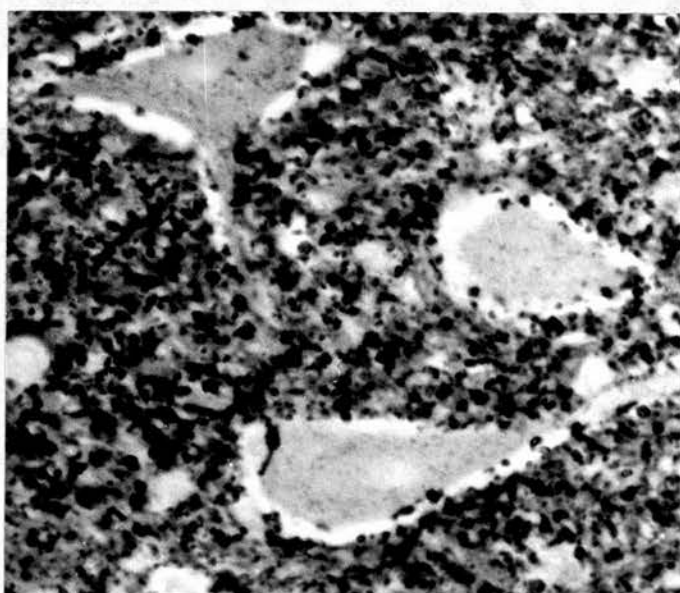
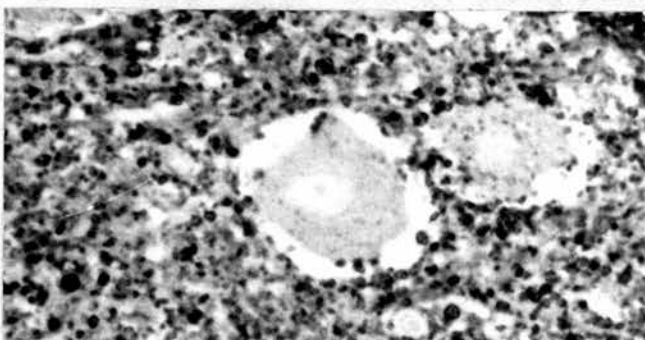


FIG. 33.



FIGS. 31-33. Hypoglossal neurones - Z10 stain - 14 days after silastic cuffs containing 0.1% vinblastine had been applied to the hypoglossal nerve. Note: reduction in numbers of somatic boutons. (cf. Figs. 6-8).

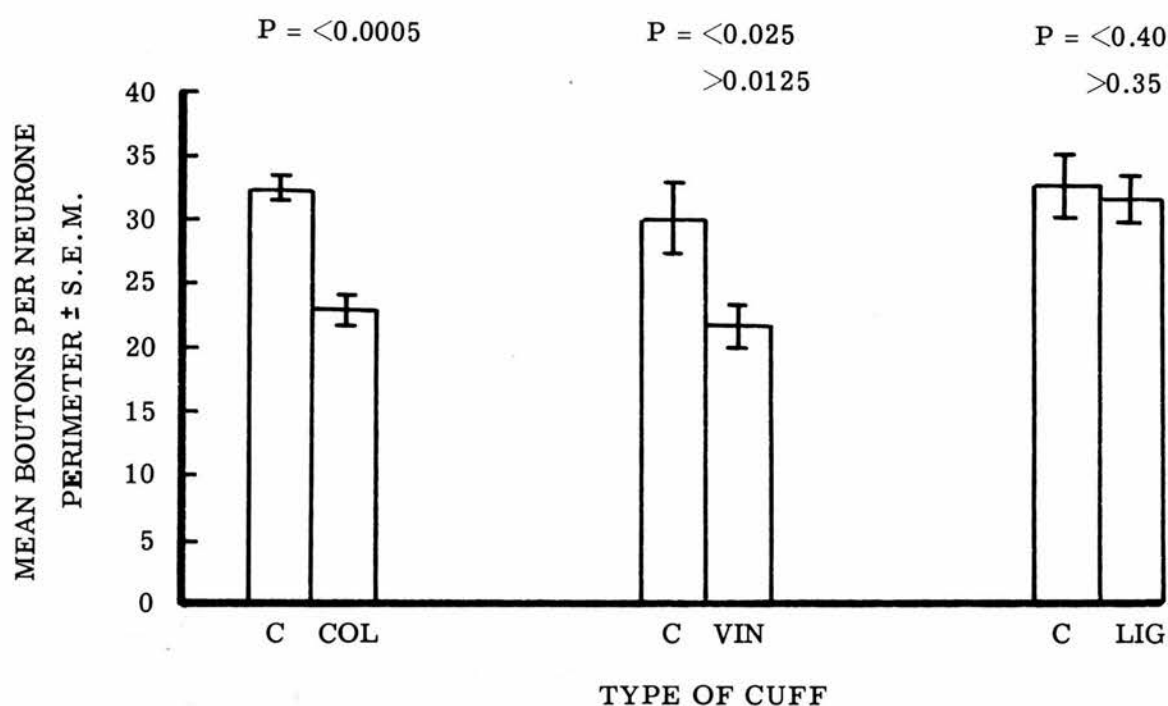


FIG. 34. Pooled data from Groups A, B, and C showing Mean boutons/neurone perimeter \pm S.E.M.. Hypoglossal nerves received :

C = Control, plain silastic cuffs.

COL = 0.1% colchicine cuffs.

VIN = 0.1% vinblastine cuffs.

LIG = 20% lignocaine cuffs.

The significance of any difference between each pair (P) is given above the columns. Note : significant reductions in bouton numbers induced by colchicine and vinblastine, but not by lignocaine.

TABLE 3

Drug/Group	Mean boutons per neurone perimeter			P
	Left	Right	L/R x 100	
A Colchicine 0.1%	25.26	33.84	74.6	<0.0005
	19.86	31.92	62.2	<0.0005
	28.30	32.82	86.2	<0.0005
	20.54	34.72	59.2	<0.0005
	21.46	31.62	67.9	<0.0005
	21.72	29.22	74.3	<0.0005
B Vinblastine 0.1%	23.44	29.64	79.1	<0.0005
	21.50	24.84	86.6	<0.005; >0.0025
	21.08	29.14	72.3	<0.0005
	26.22	40.30	65.1	<0.0005
	17.00	24.52	69.3	<0.0005
C Lignocaine 20%	26.20	26.82	97.7	<0.3; >0.25
	29.62	30.84	96.0	<0.2; >0.15
	27.74	25.54	108.6	<0.025; >0.0125
	33.66	37.90	88.8	<0.005; >0.0025
	37.74	42.24	89.3	<0.01; >0.005
	33.74	32.54	103.7	<0.3; >0.25

Axons

Compared with control nerves, axons in the drug treated areas appeared thinner and stained darker (FIGS. 35-38). This effect was most marked with colchicine cuffs. Fibre counts showed no significant difference in axon density between control and drug treated nerves (TABLE 4; FIG. 39).

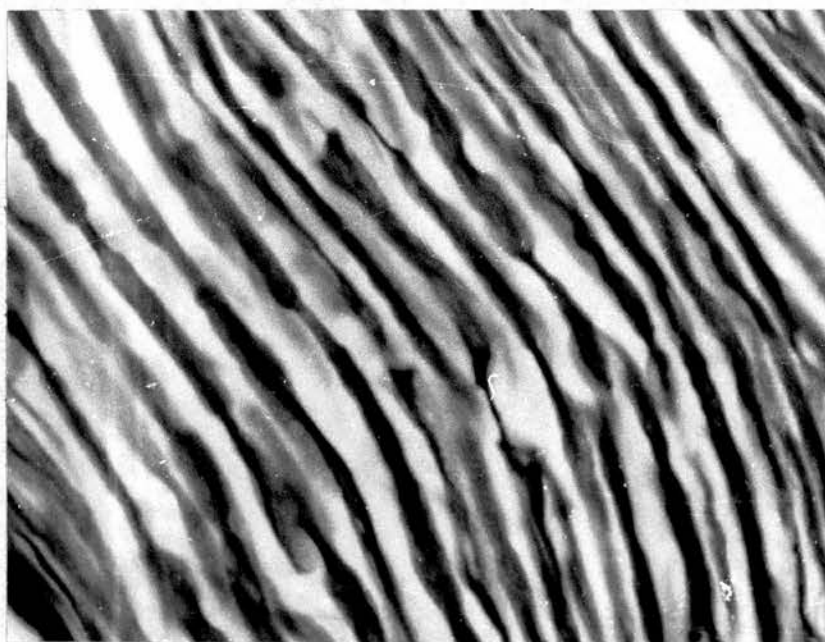


FIG. 35. Axons in a normal hypoglossal nerve, stained by Holmes' silver method.

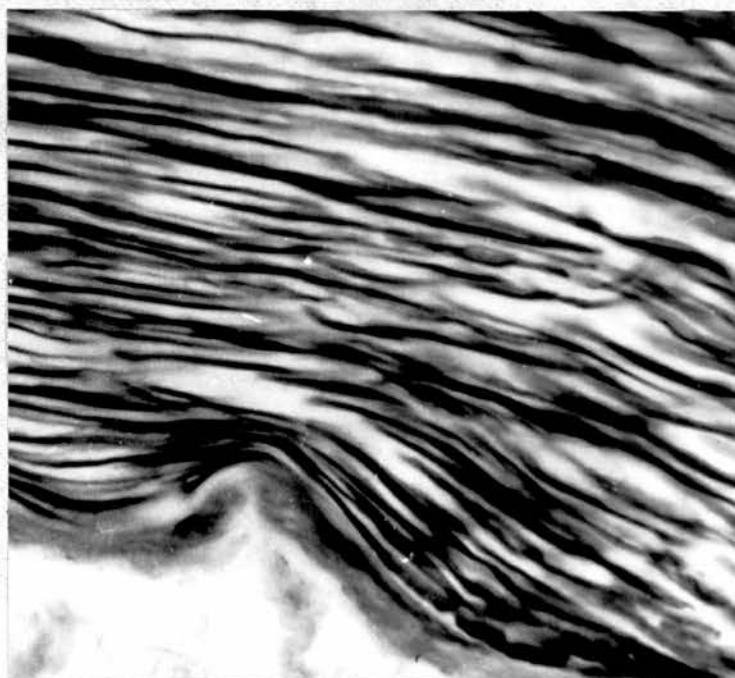


FIG. 36. Axons in a hypoglossal nerve, stained by Holmes' silver method. This portion of nerve had received a silastic cuff containing 0.1% colchicine. Note :

- (1) Slight indentation of nerve caused by cuff.
- (2) The axons stain darker and appear thinner than normal.

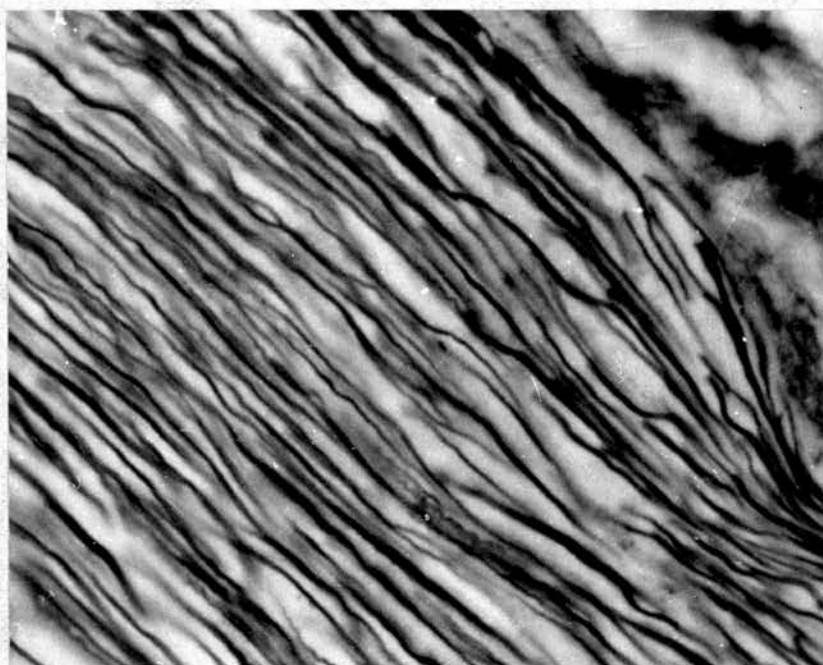


FIG. 37. Axons in the hypoglossal nerve, stained by Holmes' silver method. This portion of the nerve had received a silastic cuff containing 0.1% vinblastine. Note : axons stain darker and are thinner than normal.

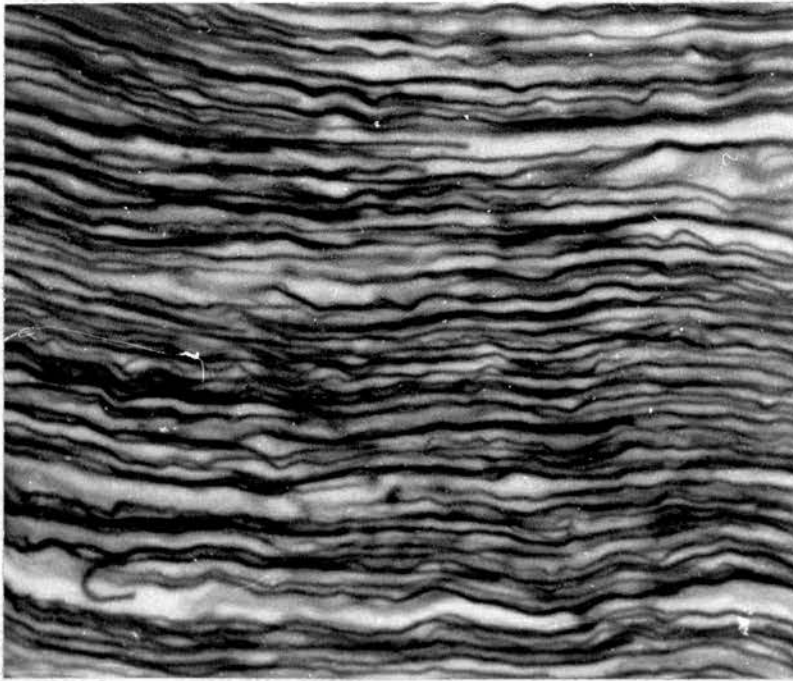


FIG. 38. Axons in the hypoglossal nerve, stained by Holmes' silver method. This portion of the nerve had received a silastic cuff containing 20% lignocaine. Note : similar, but less marked changes to those in Figs. 36 & 37.

TABLE 4

Drug/Group	Mean axons per graticule area			
	Left	+ S.E.M.	Right	+ S.E.M.
A Colchicine 0.1%	33.16	1.00	30.16	0.84
	30.00	0.75	29.83	0.81
	27.83	0.67	28.50	0.93
	29.66	1.43	28.00	0.74
	32.54	1.66	34.54	1.36
	33.90	1.85	32.90	0.97
B Vinblastine 0.1%	26.76	1.14	28.53	0.58
	30.14	0.86	28.78	0.78
	30.84	1.07	25.46	0.96
	22.75	0.86	25.30	0.43
	31.07	1.08	30.46	0.81
C Lignocaine 20%	27.28	0.57	26.07	0.64
	33.61	1.33	33.07	1.28
	29.08	0.80	28.33	0.48
	28.38	0.75	26.00	0.83
	25.92	0.66	31.38	1.29
	23.00	1.01	24.00	0.61

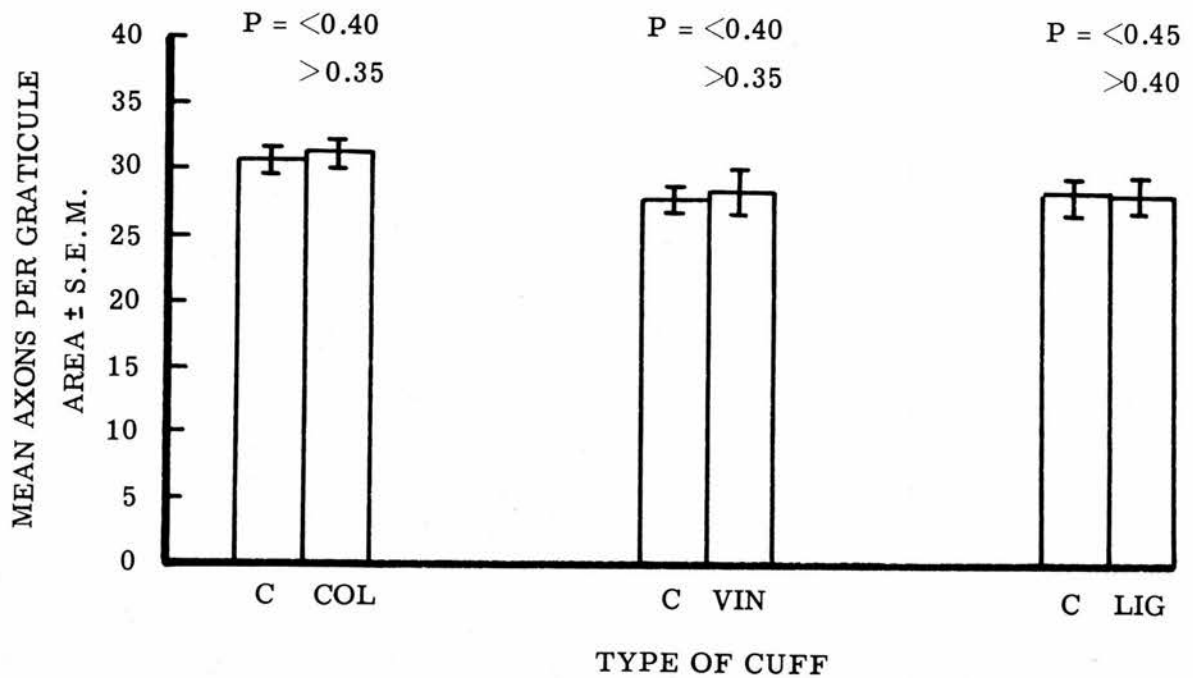


FIG. 39. Pooled data from Groups A, B and C. Axons in the hypoglossal nerves were stained by Holmes' silver method, and counted using an eyepiece with a circular graticule. Axon numbers are expressed as mean values/graticule area \pm S.E.M. . Axons counted in the area of nerve which had received :

C = Control, plain silastic cuff.

COL = 0.1% colchicine cuff.

VIN = 0.1% vinblastine cuff.

LIG = 20% lignocaine cuff.

The significance of the differences between each pair of columns (P) is shown. Note : none of the cuffs caused any significant change in axon numbers.

Nerve Conduction

Judged by the voltage of the EMG response to maximal nerve stimulation, colchicine and vinblastine cuffs did not significantly alter nerve conduction when compared with controls. Lignocaine cuffs significantly reduced the EMG voltage to maximal nerve stimuli, but complete nerve block was never achieved. (TABLE 5; FIG. 40).

TABLE 5

Drug/Group	EMG (mv) at tongue to maximal stimulus		P
	Left	Right	
A Colchicine 0.1%	12	10	$\begin{matrix} < 0.45; \\ > 0.40 \end{matrix}$
	12	14	
	20	14	
	12	16	
	10	12	
	18	16	
	<hr/> 14.00	<hr/> 13.67	
Mean			
S.E.M.	1.63	0.45	
B Vinblastine 0.1%	28	24	$\begin{matrix} < 0.475; \\ > 0.45 \end{matrix}$
	32	30	
	12	16	
	16	14	
	20	26	
	<hr/> 21.60	<hr/> 22.00	
Mean			
S.E.M.	3.71	3.03	
C Lignocaine 20%	4	15	$\begin{matrix} < 0.0025; \\ > 0.0005 \end{matrix}$
	15	35	
	10	22	
	15	35	
	20	28	
	7	33	
	<hr/> 11.83	<hr/> 28.00	
Mean			
S.E.M.	2.41	3.31	

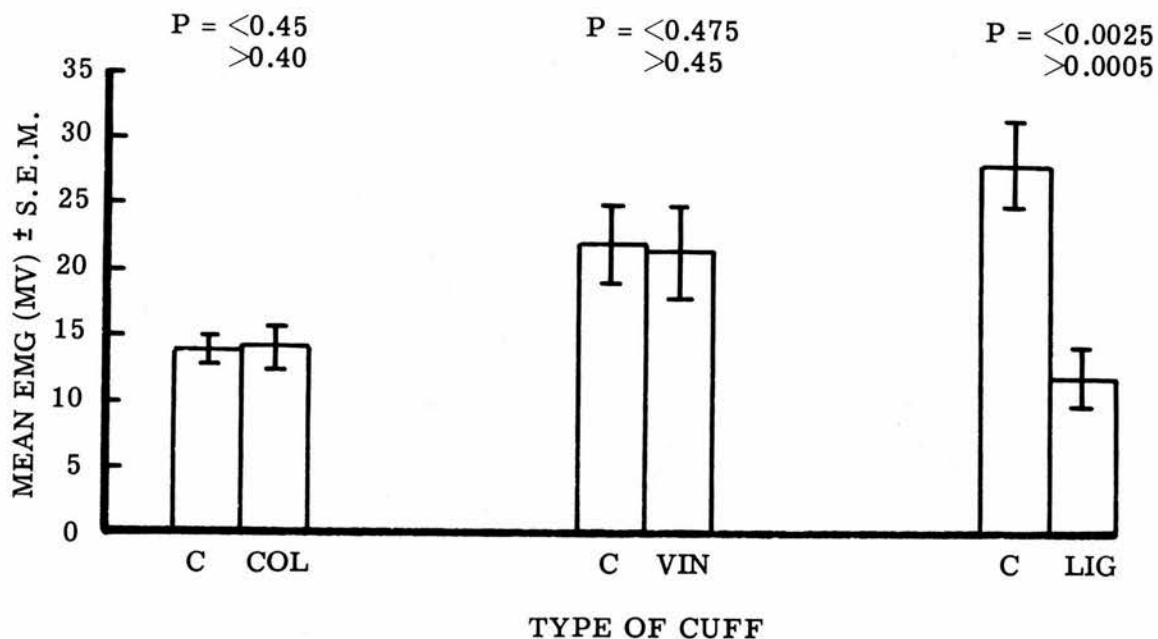


FIG. 40. EMG responses from tongue intrinsic muscles to maximal stimulation of hypoglossal nerve proximal to silastic cuffs. The nerves received :

C = Control, plain silastic cuff.

COL = 0.1% colchicine cuff.

VIN = 0.1% vinblastine cuff.

LIG = 20% lignocaine cuff.

The significance of the differences between each pair of columns (P) is shown. Note : lignocaine cuffs significantly reduced EMG responses; colchicine and vinblastine cuffs did not. The variation in control EMG values is probably due to the use of different electrodes.

Group D

Five weeks after division of their left spinal accessory nerves, rats with colchicine cuffs on their transplanted hypoglossal nerves had significantly fewer boutons per neurone perimeter on the operated side compared with plain cuff controls. (TABLE 6; FIG. 41.)

TABLE 6

Cuff	Mean boutons per neurone perimeter		
	Left	Right	L/R x 100
Plain Silastic Control	22.82	31.78	71.81
	22.94	30.52	75.16
	23.42	33.70	69.49
	20.04	31.86	62.90
		Mean	69.84 %
		S.E.M.	2.59
Colchicine 0.1%	18.34	34.58	53.04
	15.68	35.52	44.15
	20.02	34.14	58.64
	14.82	30.90	47.96
	16.28	28.38	57.36
		Mean	52.23 %
		S.E.M.	2.75
Two groups compared by Student's 't' test $P = < 0.0025; > 0.0005$			

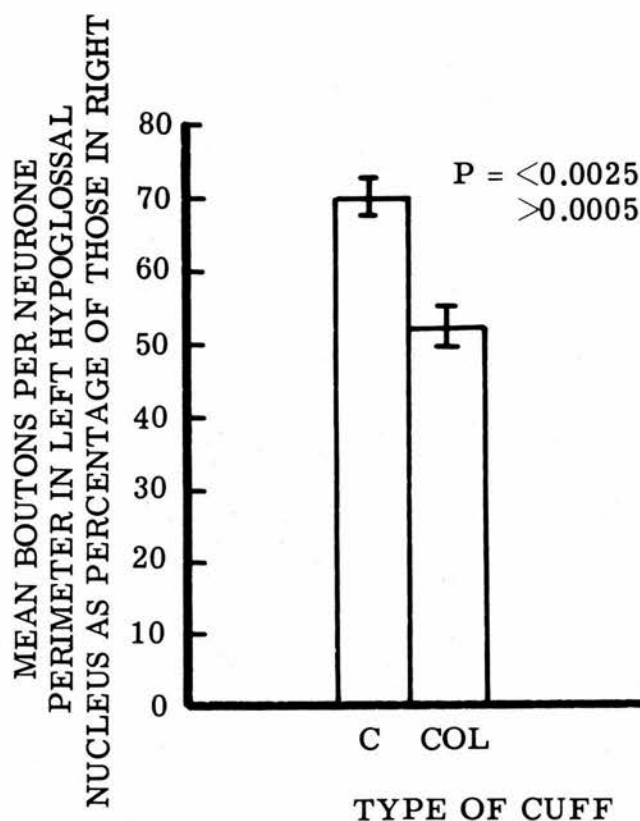


FIG. 41. Group D (see text).

Mean boutons/neurone perimeter on left side as a percentage of those on right. The left hypoglossal nerve had been implanted into the sternomastoid muscle; and, after the spinal accessory nerve had been divided, the hypoglossal nerve received either :

C = Control, plain silastic cuff.

COL = 0.1% colchicine cuff.

Note : restoration of bouton number is inhibited significantly by colchicine.

DISCUSSION

Silicone rubber (silastic) is very pliable at 37°C, and causes little reaction when implanted into mammalian tissues (187-188). Vulcanised silastic takes up dyes from aqueous solutions and releases them slowly when implanted (189). Solid drugs mixed with liquid silastic before vulcanisation are released gradually after the solidified mixture is implanted (189). Silastic implants containing tritiated colchicine release about 40% of the drug over the first five days (190); a cuff might thus continue to release colchicine for about 2 weeks.

Silastic cuffs, much larger than those in the present study, have been used to apply drugs to rat sciatic nerves (44; 51-52; 190). The relative size of the rat hypoglossal nerves made it necessary to use much smaller implants for the present experiments. It is estimated that the cuffs used contained one of : 10 µg colchicine; 10 µg vinblastine; or 2 mg Lignocaine. Rat sciatic nerves, treated with colchicine or vinblastine cuffs, continue to transmit impulses normally; but the muscles supplied by the nerves develop electrophysiological signs of denervation (52). Muscle transmembrane potential falls; the area receptive to applied acetyl choline increases; and tetrodotoxin-resistant action potentials appear (52). Muscles behave similarly if their motor nerves are cut (42-43). Cuffs containing 20% lignocaine do not cause changes of denervation despite causing blockage of impulse transmission (51). Cuffs containing

25% bupivacaine - a local anaesthetic about four times as potent as lignocaine - do cause some signs of muscle denervation, however (44).

The present study indicates that, when hypoglossal nerves are treated with colchicine and vinblastine for two weeks, synaptic boutons are lost from the surfaces of parent neurones. A similar effect probably occurs in autonomic ganglia, since treatment of the post-ganglionic trunk of the ciliary ganglion with colchicine causes failure of the trans-synaptic response to afferent nerve stimulation (191). Cuffs containing 20% lignocaine have little or no effect on hypoglossal neurone boutons; but, since nerve block was incomplete, the dose may have been too small. Although the effects of colchicine and vinblastine cuffs could be due to axonal injury, no changes in axon numbers were found; and no significant decreases in EMG voltage were detected.

Colchicine and vinblastine inhibit both anterograde (107; 109; 115; 118) and retrograde axonal transport (39; 115; 117). Injection of 10 μ g of colchicine into a rabbit's eye inhibits both fast and slow axonal transport rates; after 47 days, there is still about 30% inhibition (109). The concentrations of colchicine and vinblastine achieved at the hypoglossal nerve by the present cuffs are not known; but they are likely to have been high enough to inhibit axonal transport. Further experiments have confirmed this (See Section 7 (d).)

Colchicine and vinblastine inhibit the rapid movements of intracellular organelles in cultured cells (99-102),

and prevent cell division by stopping the formation of mitotic spindles (119). Cells bind colchicine in proportion to their richness in microtubules (125); those with mitotic spindles or cilia have the highest affinity. The molecule which binds colchicine is a dimer of the protein tubulin (molecular weight 60,000 daltons) (124; 126). Cells and axons treated with colchicine or vinblastine show loss of microtubules (95; 97; 99-102; 107; 109; 118-121). Both drugs bind with high affinity to tubulin (117; 126). Since microtubules are probably involved in axonal transport (see Introduction), they would appear to be the sites at which the drugs act.

The present evidence thus suggests that inhibition of axonal transport causes loss of synaptic boutons on parent neurones. Nerves both release and take up proteins at their terminals (29-32; 39; 56-57) and there is growing evidence that material may be transferred from cell to cell across synapses (66). In the system under study here, it is proposed that loss of material, which would normally come from muscle and be transported to the perikaryon, causes hypoglossal neurones to shed synaptic boutons. The failure of boutons to return after application of a colchicine cuff (Group D) to the transplanted nerves supports this; but the drug might be impeding muscle innervation by inhibiting axon growth. Injection of a large dose (300 μ g) of colchicine into a divided nerve does reduce axonal sprouting (192); but this effect is not likely to be occurring here, since 0.1% colchicine cuffs do not slow the return of crashed hypoglossal axons to the tongue

(see below; Section 8).

The effects of local anaesthetics are less clear. 20% lignocaine cuffs do not induce changes of denervation in muscles, despite causing a full conduction block (51); but 25% bupivacaine cuffs cause the area of muscle responsive to acetyl choline to spread (44). Procaine does not interfere with axonal transport in vitro (193); but lignocaine has been reported to reduce microtubule numbers and inhibit axonal transport in vitro (194). The effects of colchicine and vinblastine on muscle properties and synaptic boutons seem to relate to inhibition of axonal transport; and it is likely that high concentrations of local anaesthetics have similar effects. The present study showed that lignocaine cuffs induced a small reduction in bouton numbers in only two of the six animals. Overall, the group of rats did not show significant bouton loss; neither was retrograde axonal transport significantly inhibited (See Section 7 (d)).

These results indicate that drugs which inhibit axonal transport cause loss of synaptic boutons on parent neurones when applied to their axons. Nerve conduction block with local anaesthetics does not cause significant bouton loss. It is suggested that colchicine and vinblastine deprive the perikaryon of factors normally derived from muscle. Effective nerve-muscle contact may be required for the uptake of such factors; this possibility is investigated below (sections 6 and 7).

6. EFFECTS OF SALINE AND OF MUSCLE EXTRACTS

METHODS

Operations

Three groups of rats were studied:

Group A contained 10 rats. The left hypoglossal nerve was exposed as in Sections 2 and 3 and transfixed with a fine silk suture, which was tied with a loose knot. The nerve was then divided a few mm distal to the suture, and the central stump gently pulled into the lumen of a 3-4 cm length of silastic tubing (internal diameter 0.25 mm ; external diameter 0.5 mm). This was done by passing the silk down the tubing before transfixing the nerve; and then pulling the free end of the suture until about 1 cm of nerve had entered the tube. The tube was then filled with fluid extracted from normal muscle (see below), and the free end sealed with double sutures. The tubing containing the central nerve stump and extract fluid was then buried in the interstitial tissue of the neck, and the wound was closed. (See FIGS. 42-45).

Group B contained 10 rats. The left hypoglossal nerve was treated as in Group A, but the silastic tube was filled with fluid extracted from denervated muscle (see below).

Group C contained 5 rats. The left hypoglossal nerve was treated as in Groups A and B, but the silastic tube was filled with sterile 0.9% aqueous sodium chloride solution (saline).

Preparation of muscle extracts

Normal muscle extract (Group A).

Two rats were killed by stunning (no anaesthetic was

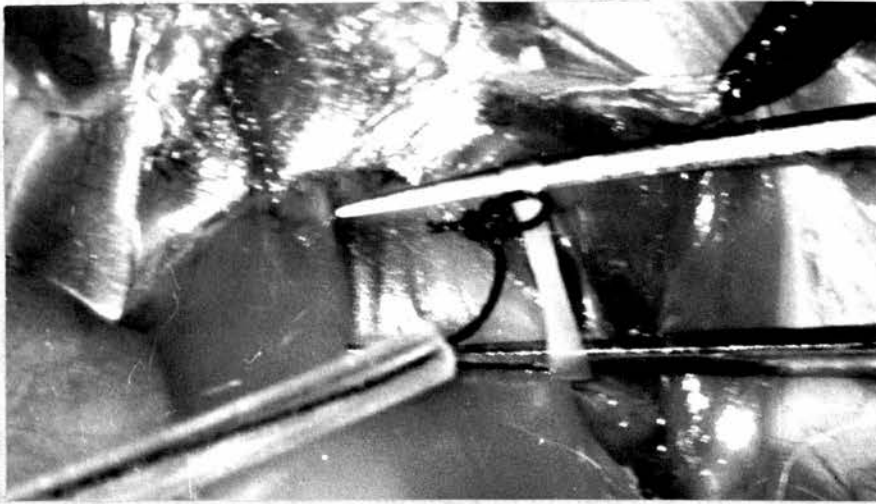


FIG. 42. The left hypoglossal is transfixed with a silk suture, which is then tied with a loose knot.

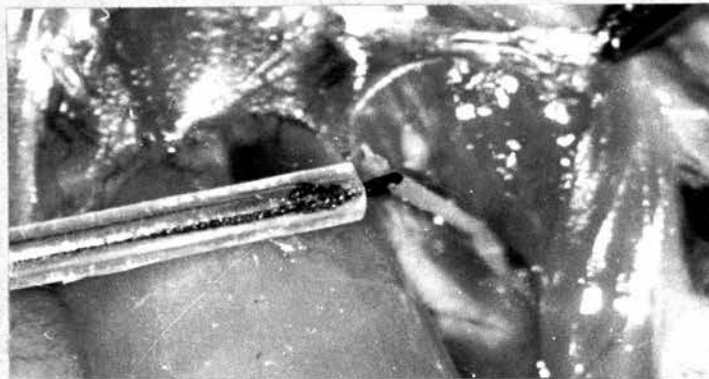


FIG. 43. The nerve is divided distal to the suture, and its central end is pulled into a piece of silastic tubing.



FIG. 44. The central stump of the hypoglossal nerve inside the silastic tube.

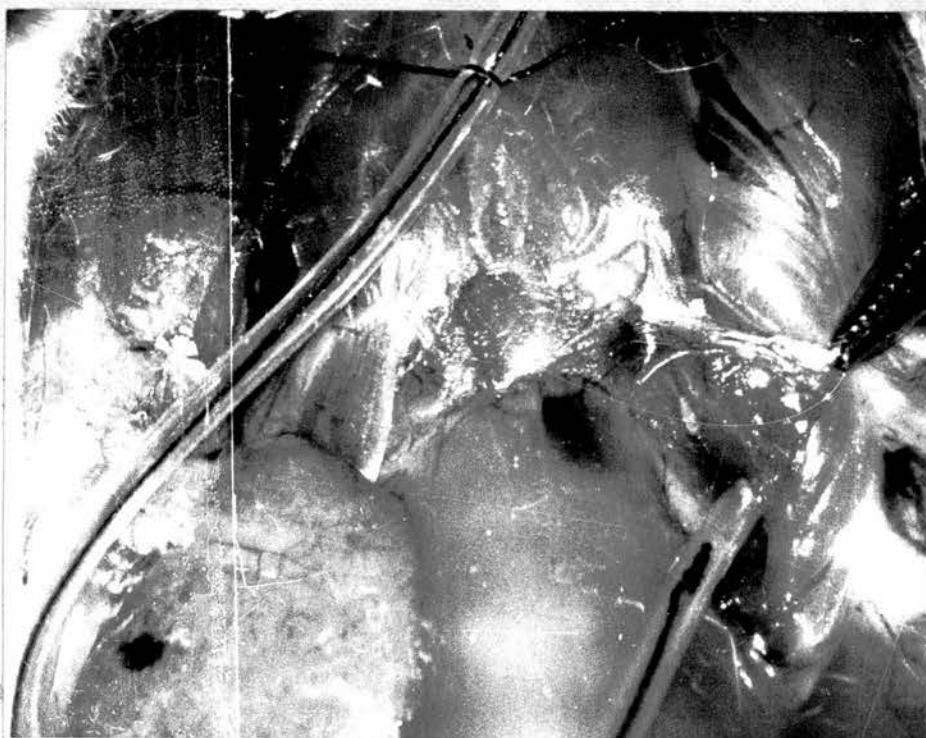


FIG. 45. The silastic tube is filled with fluid (saline or muscle extract); sealed at its far end with silk ligatures; and buried in the interstitial tissue of the neck.

used) and perfused through the left cardiac ventricle with 100 ml 0.9% aqueous sodium chloride. The hamstring and calf muscles were removed from both legs and frozen until very firm. The muscle was then chopped into small slices with a scalpel blade until a fine mince was obtained. This was refrozen, and the process repeated to a total of five freezings. The muscle mince was then packed into 5 ml plastic centrifuge tubes and spun at 30,000 RPM for 1 hour. The supernatant fluid was removed and left at 4°C overnight; recentrifuged at 3,000 RPM for 1 hour and the clear supernatant kept at 4°C until use.

Denervated muscle extract (Group B).

The sciatic nerves of 2 rats were divided high in the thigh. Four days later, the rats were killed and their hamstring and calf muscles used to make an extract as above.

Both types of muscle extract were passed through bacterial filters (Millipore UK Ltd. GSWP 01300. Pore size 0.22 μ m) before use.

Histology

7 days after implantation of silastic tubes, rats were killed and their medullae impregnated in Z10 as in section 2 (a).

Analysis of numerical data

Boutons were counted on 100 hypoglossal neuronal profiles from each animal as before. 50 profiles were

assessed from each hypoglossal nucleus. Slides were double coded as in Section 4, so that the observer did not know from which group of rats a slide came.

Bouton counts from the three groups were compared by Student's 't' test. 500 neuronal profiles pooled from the left hypoglossal nucleus of Group A were compared with 500 profiles from Group B and 250 from Group C. Similarly, Groups B and C were compared. (See FIG. 46).

Bouton counts from the right hypoglossal nuclei of the three groups were similarly compared. (See FIG. 47).

Averaged data, standard errors and percentages of bouton losses are shown in TABLE 7.

RESULTS

The mean numbers of boutons on the left (operated) sides of groups A and B did not differ significantly. But bouton numbers on the operated sides of saline treated rats (Group C) were significantly lower than those in both the muscle extract groups ($P = <0.0005$) (FIG. 46). Comparison of the unoperated right sides of the three groups showed that there were significantly more boutons on the control sides of Group B rats ($P = <0.0005$) (FIG. 47).

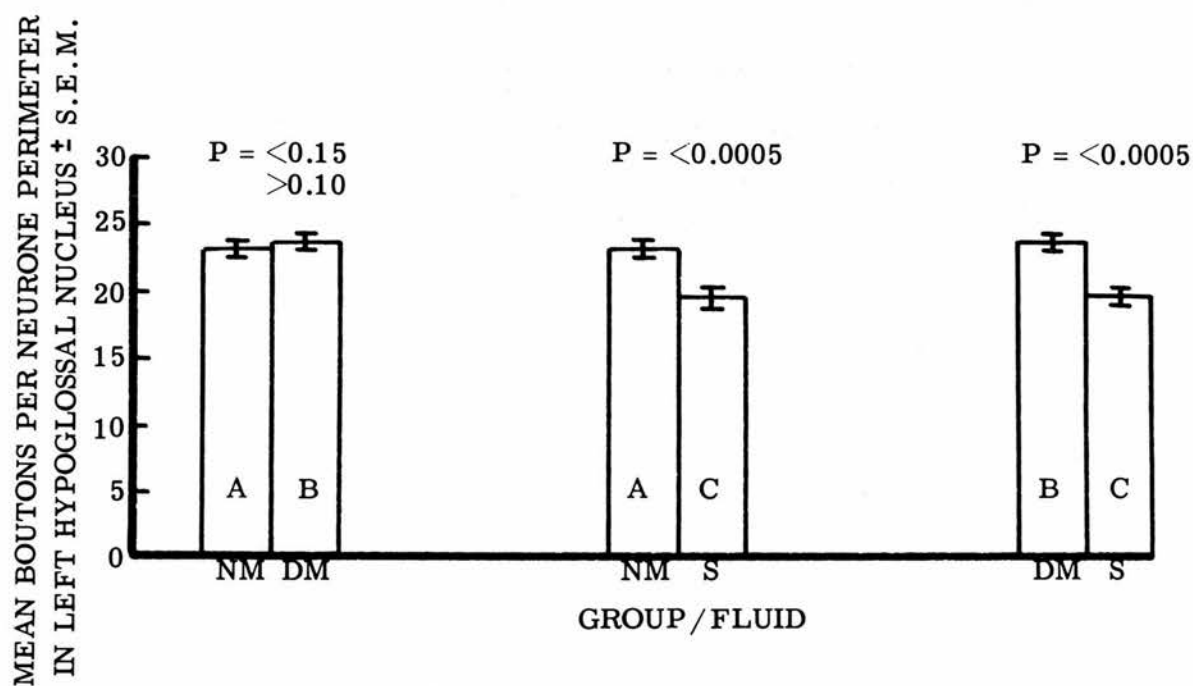


FIG. 46. Mean boutons/neurone perimeter in the left hypoglossal nucleus one week after the left hypoglossal nerve was divided and its central end bathed in :

NM = Normal muscle extract.

DM = Denervated muscle extract.

S = 0.9% sodium chloride solution (saline).

Note : significantly fewer boutons on neurones whose axons were bathed in saline.

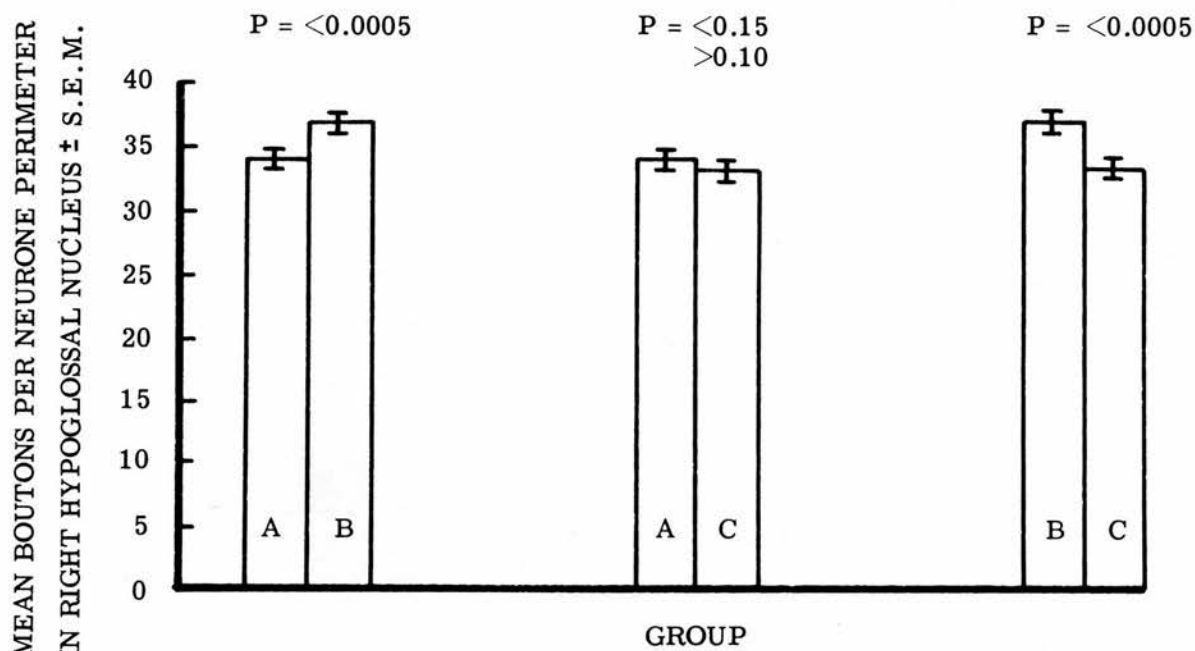


FIG. 47. Mean boutons/neurone perimeter in right (control) hypoglossal nuclei of Groups A, B and C.

Note : Group B have significantly more boutons than Groups A and C. This is probably caused by differences in stain penetration.

TABLE 7

Group/Fluid	Mean boutons per neurone perimeter $\frac{L}{R} \times 100$				
	Left	S.E.M.	Right	S.E.M.	
A Normal Muscle extract	23.12	0.36	34.08	0.35	67.83%
B Denervated muscle extract	23.65	0.36	36.90	0.41	64.09%
C Normal Saline	19.55	0.37	33.34	0.51	58.64%

DISCUSSION

These experiments were designed to investigate the hypothesis that axotomy and inhibitors of axonal transport cause neurones to shed boutons because they interrupt the passage of macromolecules from muscle to neuronal perikarya. Watson had found that injections of extracts of normal and denervated muscle into sternomastoid muscles bearing transplanted hypoglossal nerves caused different patterns of nucleolar response in hypoglossal neurones. (Watson, W.E.; unpublished results). But it was unclear whether material in the extracts was acting on the normal muscle, on the implanted hypoglossal nerve, or the normal accessory nerve. It was therefore of interest to see whether extracts of normal and denervated muscle could protect neurones from

bouton loss after axotomy.

The results are difficult to interpret. Both normal and denervated muscle extracts appear to promote bouton retention equally well when compared with saline. But, comparing the percentage bouton loss in the three groups with that induced by simple axotomy (see TABLES 1 and 2), it seems that the saline treated group has more bouton loss than rats 7 days after axotomy alone. The rats treated with muscle extracts do not show any convincing bouton restoration when compared with axotomy alone; it is rather that saline exacerbates bouton loss.

The higher bouton numbers on the control sides of Group B rats is difficult to explain. It is unlikely to have been due to the extract; and was more likely caused by differences in stain penetration.

Failure of the muscle extracts to prevent bouton loss may have been due both to loss of important material during the extraction procedure, and to failure of axonal uptake. The chemical composition of the muscle extracts is not known. It is likely that much of the protein was denatured during preparation; many membrane-related proteins were probably removed by centrifugation. More careful and selective muscle preparations may be needed to achieve meaningful results.

The growth cones of cultured sympathetic neurites take up extracellular proteins by pinocytosis (209). But uptake of tracer protein by axons has been very poor (see below; Section 7 (c)). It is possible that neuronal bouton loss after axotomy causes the axon tips to take up

less extracellular material, since the amount of uptake is known to depend on the frequency of nerve action potentials (30; 195).

The only clear effect shown by this study was the increased bouton loss induced when cut axons were bathed in saline. Both denervated and normal muscle extracts appear to protect neurones from this effect; but they are little better than the normal tissue fluids likely to be encountered by regenerating axons. Nerves with motor end-plates take up exogenous protein much more avidly than growing axon tips (see below; section 7 (a-c)); thus effective nerve-muscle contact may maintain synaptic connections by providing an efficient uptake mechanism for muscle-derived materials.

7. RETROGRADE AXONAL TRANSPORT OF HORSE RADISH PEROXIDASE IN:

(a) NORMAL AND REGENERATING NERVES

METHODS

Operations

41 rats were studied at intervals between 4 and 22 days after the left hypoglossal nerve had been crushed as in 3 (a). On the day before death, rats were anaesthetised with ether, and 0.1 ml of a solution containing 50 mg/ml horseradish peroxidase (HRP) in 0.2 M phosphate buffer at pH 7.4 was injected into the intrinsic muscles on each side of the tongue. 24 hours later, rats were anaesthetised with urethane and both hypoglossal nerves were exposed as they passed deep to the posterior belly of the digastric muscle. The voltages of EMG responses to maximal nerve stimuli were recorded from the intrinsic tongue muscles as in section 5. The needle electrode was inserted into approximately the same regions that had been injected with HRP.

After electrophysiological measurements had been made, animals were perfused through the left cardiac ventricle with a pre-wash of 20 ml 0.2 M phosphate buffer (pH 7.4) containing 1% gum acacia; and were then perfused with 40 ml of a modified Karnovsky fixative (196). This was made by dissolving 4 g paraformaldehyde and 1 g gum acacia in 50 ml distilled water at 60°C, and adding 6 drops of 1N sodium hydroxide. The solution was cooled, and then 10 ml 50% glutaraldehyde added. The mixture

was made up to a total volume of 100 ml with 0.2 M phosphate buffer (pH 7.4). Pre-wash and fixative solutions were chilled to 4°C before use; they were made up fresh each day. After this perfusion, the medulla oblongata was removed as in 2 (a) and transferred to 10 ml of the above fixative at 4°C.

Histology

After about 4 hours in fixative, medullae were transferred directly to a freezing microtome, and 30 μ m transverse sections were cut at 200 μ m intervals through the block. Sections were received into water and washed for 30 minutes. They were then transferred to 75 ml of a solution containing 0.08% diaminobenzidine in 1/30 M phosphate buffer at pH 7. After 10 minutes, 0.25 ml of 20 vols. hydrogen peroxide were added, and the sections left for a further 10 minutes. The sections were then dehydrated for five minutes in each of 70%; 90%; and absolute alcohols, cleared for 5-15 minutes in xylene and mounted in DPX.

Microscopy and analysis

Sections were viewed under phase contrast at X400 magnification. The number of neurones showing uptake of HRP was counted in each hypoglossal nucleus. About 10 sections were analysed from each animal. The mean number of HRP positive cells per section on the left side was compared with that on the right and expressed as a percentage $\frac{\text{LEFT}}{\text{RIGHT}} \times 100$. Means for each day and standard errors are shown in TABLE 8 and FIG. 48. Days after axotomy are measured to the time of HRP injection.

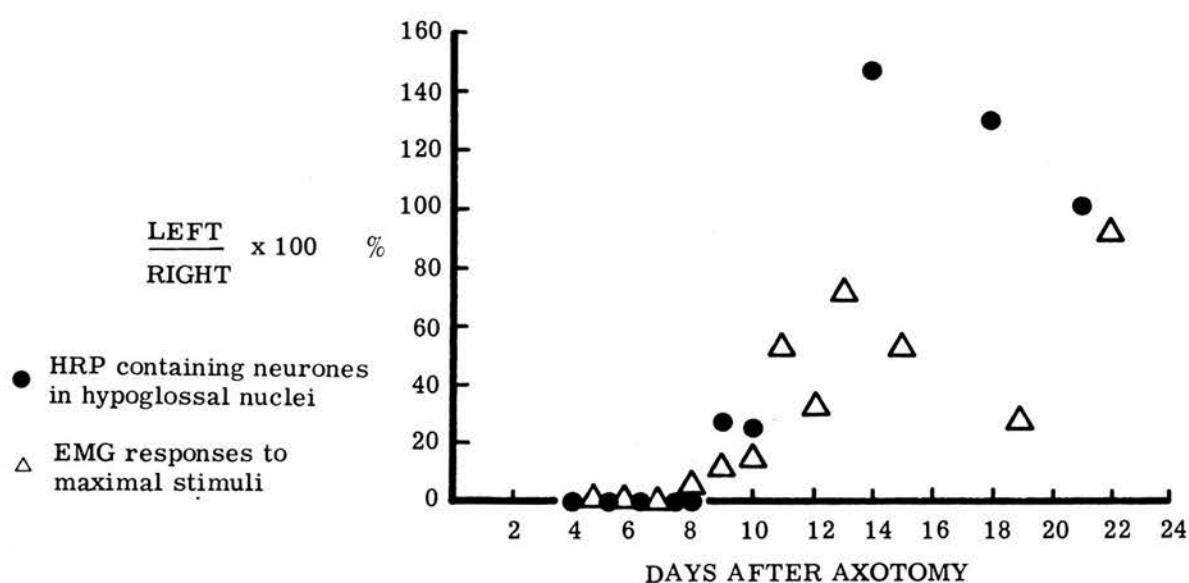


FIG. 48. The number of HRP-containing hypoglossal neurones/section (dark circles), and the EMG responses recorded from the tongue (triangles) are expressed as percentages $\frac{\text{Left}}{\text{Right}} \times 100$, and plotted against days after left hypoglossal nerve crush. Note :

- (1) HRP uptake and EMG return almost simultaneously.
- (2) HRP uptake becomes greater than normal during early reinnervation of muscle.

RESULTS

Peroxidase uptake

Neurones that had taken up HRP were identified by fine dark granules present in their cytoplasm. These granules were most readily seen under phase contrast. (See FIGS. 49-50). Neurones from animals which had not received HRP injections did not show similar granules (FIGS. 51-52). The number of hypoglossal neurones on the control (right) sides which showed HRP uptake varied from zero to about 20 per section.

After axotomy, HRP uptake was absent on the operated side; but it began to return on the ninth day. Between the 14th and 18th days after axotomy, the number of stained cells on the regenerating side exceeded that on the right, but subsided to normal again by 3 weeks. (TABLE 8; FIG. 48).

EMG responses

The EMG voltages to maximal stimuli are expressed as $\frac{\text{Left side}}{\text{Right side}} \times 100$ and are shown in TABLE 8 and FIG. 48. Days after operation are measured to the time of EMG recording. Axotomy abolished the EMG response on the operated side until the eighth day after operation, when a delayed, polyphasic response was seen. Thereafter, the responses increased progressively with time, and were near normal by three weeks.

FIG. 49.

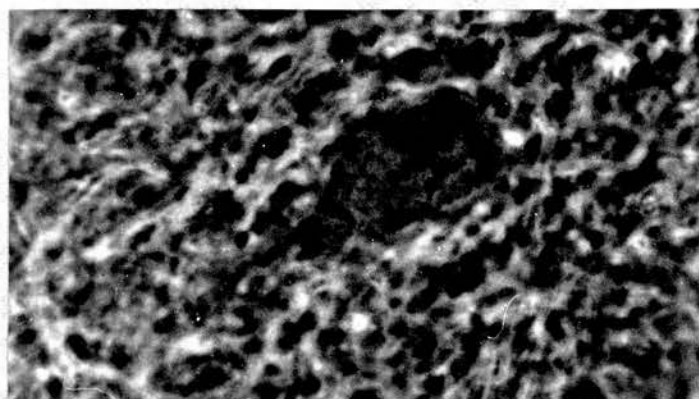
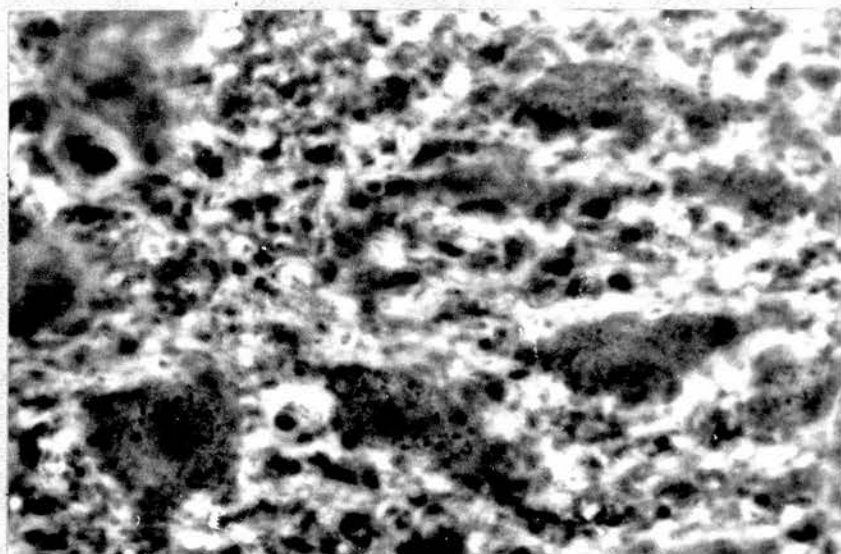


FIG. 50.



FIGS. 49 & 50. Phase contrast photomicrographs of hypoglossal neurones showing uptake of HRP. The protein appears as dark granules within the neuronal cytoplasm. (cf. Figs. 51 & 52).

FIG. 51.

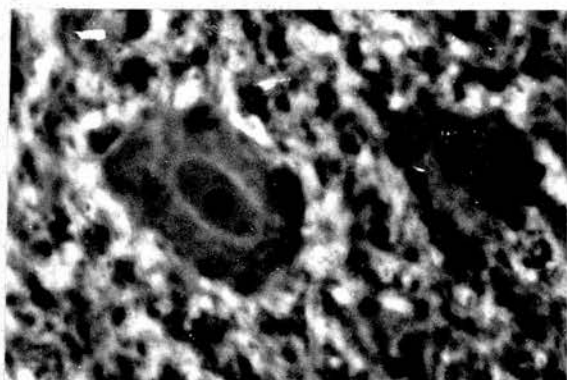
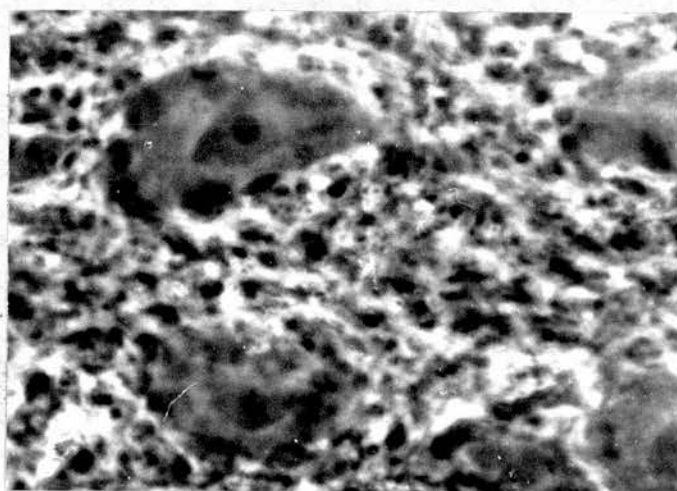


FIG. 52.



FIGS. 51 & 52. Phase contrast photomicrographs of normal hypoglossal neurones stained for peroxidase. (No HRP injected). Note : absence of dark granules in neuronal cytoplasm (cf. Figs. 49 & 50).

TABLE 8

Days after axotomy	EMG response to max. stimulus		Mean number of HRP -con- taining neurones/section	
	LEFT $\times 100$ RIGHT	S.E.M.	LEFT $\times 100$ RIGHT	S.E.M.
4	-	-	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	2.2	1.4	0	0
9	12.1	5.2	27.6	13.3
10	17.1	4.0	25.7	25.7
11	53.0	17.5	-	-
12	33.3	3.1	-	-
13	71.5	3.5	-	-
14	-	-	147.0	65.0
15	53.5	13.5	-	-
18	-	-	130.5	1.5
19	29.0	4.0	-	-
21	-	-	101.0	-
22	91.5	41.5	-	-

(b) TRANSPLANTED NERVES

METHODS

Operations

Six rats were divided into two groups.

Group 1 contained 4 rats. The left hypoglossal nerve was divided and transplanted into the ipsilateral sternomastoid muscle, as in Section 3 (a). One day (1 rat) or two months (3 rats) later, the neck was reopened and 10-15 mg of HRP in 0.2 M phosphate buffer (pH 7.4) injected into the left sternomastoid muscle, close to the implanted nerve. 24 hours later perfusion fixation was carried out as in Section 7 (a), and the medullae and upper cervical spinal cords removed for histology.

Group 2 contained two rats. The left hypoglossal nerve was transplanted as above, and two months later the left spinal accessory nerve was divided. Eleven days later, 10 mgs of HRP was injected into the left sternomastoid muscle, as above. After a further 24 hours, the rats were fixed by perfusion and their medullae removed for histology.

Histology

Fixed tissue was treated exactly as in Section 7 (a). About 10 sections from each animal were examined under phase contrast for HRP containing cells. Detailed numerical analysis was not made.

RESULTS

Group 1 Rats whose left hypoglossal nerves had been implanted into normally innervated sternomastoid muscles showed no uptake of HRP into hypoglossal neurones. In 3 rats, where sections of upper cervical cord had been made, a few anterior horn cells showed HRP uptake. These were likely to have been spinal accessory neurones.

Group 2 Some hypoglossal neurones whose axons lay in denervated sternomastoid muscles showed uptake of HRP; but only 3 such positive cells were seen in 20 sections. In both rats, 4-5 hypoglossal neurones on the right side also showed HRP uptake.

(c) NERVES IN SILASTIC TUBES

METHODS

Operations 12 rats were treated as follows : the left hypoglossal nerve was divided, and its central stump led into a silastic tube (as in section 6) which contained HRP, 50 mg/ml in 0.2 M phosphate buffer at pH 7.4. Between one and seven days later, rats were fixed by perfusion and their medullae removed.

Histology was carried out as in Section 7 (a) and (b).

RESULTS

Animals fixed one day after operation (4 rats) showed no uptake of HRP in hypoglossal neurones. Those fixed 3-7 days after operation (8 rats) showed granular HRP uptake in a small proportion of their left hypoglossal neurones. Between 2 and 4 days, HRP containing cells were seen in every 20 sections examined.

(d) NERVES TREATED WITH DRUG-IMPREGNATED

SILASTIC CUFFS

METHODS

Operations 12 rats were divided into three groups of four animals.

Group 1. Silastic cuffs containing 0.1% colchicine were applied to the left hypoglossal nerve, as in section 5; plain silastic cuffs were applied to the right hypoglossal nerves.

Group 2. 0.1% vinblastine cuffs were applied to the left, and plain cuffs to the right hypoglossal nerves.

Group 3. 20% lignocaine cuffs were applied to the left, and plain cuffs to the right hypoglossal nerves.

14 days later, all groups received 5 mg HRP in 0.2 M phosphate buffer (pH 7.4), injected into each side of the tongue.

Histology 24 hours after receiving peroxidase, rats were anaesthetised with ether and the cuffed regions of the hypoglossal nerves removed. Nerve pieces were about 1 cm long. Perfusion fixation was then carried out, and the medullae removed.

Medullae were treated as in Section 7 (a). Slides from the three groups were double coded, so that they could be assessed "blind". The numbers of HRP containing neurones were counted in the left and right hypoglossal nuclei of each section; the results for each group were pooled and the two sides compared by student's 't' test.

Nerves were fixed for one week in 10% formol-saline.

They were then stained for degenerate myelin by the method of Swank and Davenport (186). After fixation, nerves were transferred directly to a solution containing : 1% potassium chlorate (6 ml); 1% osmium tetroxide (2 ml); glacial acetic acid (0.1 ml); 40% formalin (1.2 ml). They were left in this for 1 week and agitated daily. Then, after washing in water for 24 hours, nerves were dehydrated in 15 minute changes of 70%; 90% 95% and Absolute alcohol, cleared in two 15 minute changes of benzene, embedded in three 30 minute baths of paraffin wax and cut along their long axis into 30 um sections. These were mounted on glass slides, cleared in xylene and mounted in DPX.

Microscopy was carried out at X100 under transmitted light. The number of nerve fibres showing myelin degeneration was counted for each section; ten sections were examined from each nerve. Pooled results were analysed by student's 't' test, to compare drug treated nerves with controls.

RESULTS

Peroxidase uptake

Pooled results showing mean numbers of HRP-containing neurones per section in the left and right hypoglossal nuclei, standard errors and the significance of their differences are shown in TABLE 9 and FIG. 53. Colchicine and vinblastine cuffs reduced significantly the number of neurones taking up HRP. Lignocaine cuffs caused smaller reductions in HRP uptake which did not reach statistical significance.

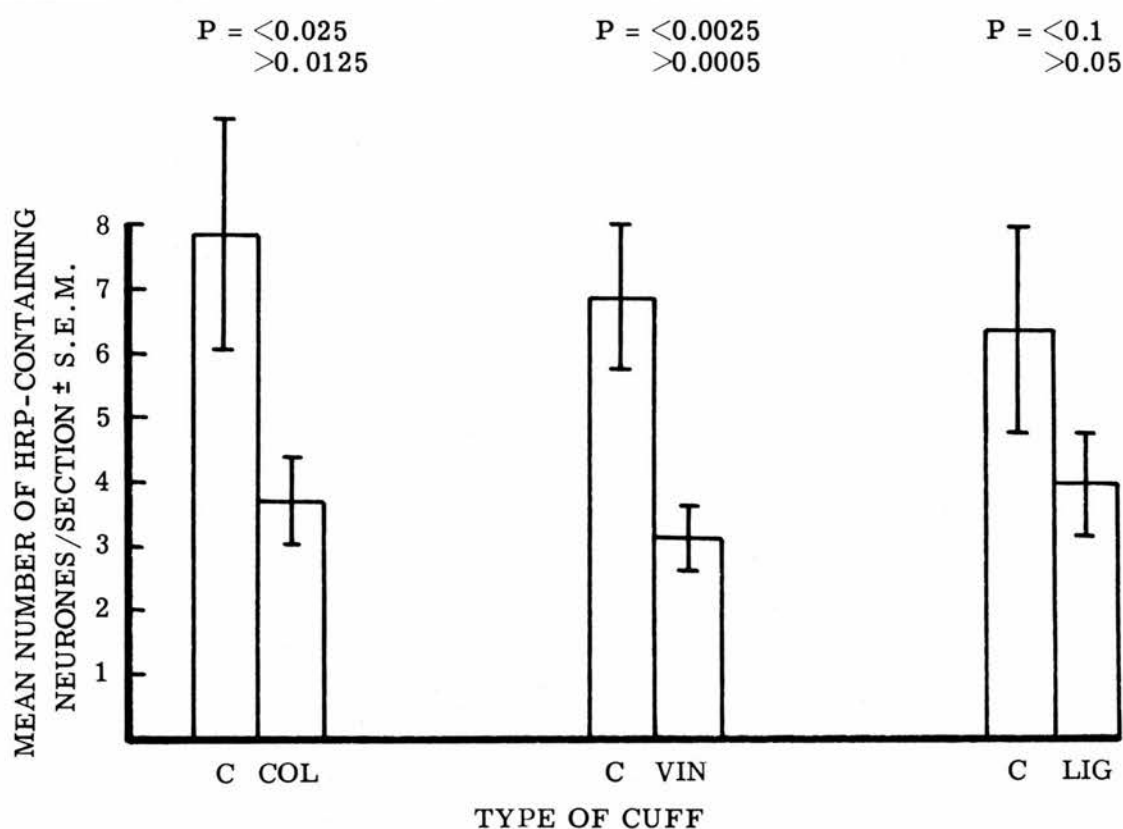


FIG. 53. Columns showing mean number of HRP-containing hypoglossal neurones/section, with standard errors. The hypoglossal nerves received :

C = Control, plain silastic cuff.

COL = 0.1% colchicine cuff.

VIN = 0.1% vinblastine cuff.

LIG = 20% lignocaine cuff.

Note : colchicine and vinblastine cuffs caused significant inhibition of retrograde transport of HRP. The inhibition due to lignocaine was not significant.

TABLE 9

Group/Drug	Total Sections Examined	Mean number of HRP containing neurones/section						P
		Left	S.E.M.	Right	S.E.M.	$\frac{\text{Left}}{\text{Right}} \times 100$		
1 0.1% Colchicine	19	3.7	0.7	7.8	1.8	46.9		< 0.025 > 0.0125
2 0.1% Vinblastine	25	3.1	0.5	6.8	1.2	45.0		< 0.0025 > 0.0005
3 20% Lignocaine	17	3.9	0.8	6.3	1.6	62.6		< 0.1 > 0.05

Nerve fibres with degenerate myelin

Fibres with degenerate myelin stained black against a pale yellow background of the rest of the nerve. Results pooled from each group of animals are shown in TABLE 10 and FIG. 54. The mean number of fibres with degenerate myelin per section, standard errors and significance of differences are shown for the right and left hypoglossal nerves of each drug group. Nerves treated with colchicine cuffs showed a significant increase in myelin degeneration compared with controls. Vinblastine and lignocaine did not cause any significant increase in myelin degeneration. Photomicrographs of control and colchicine treated nerves are shown in FIGS. 55-56.

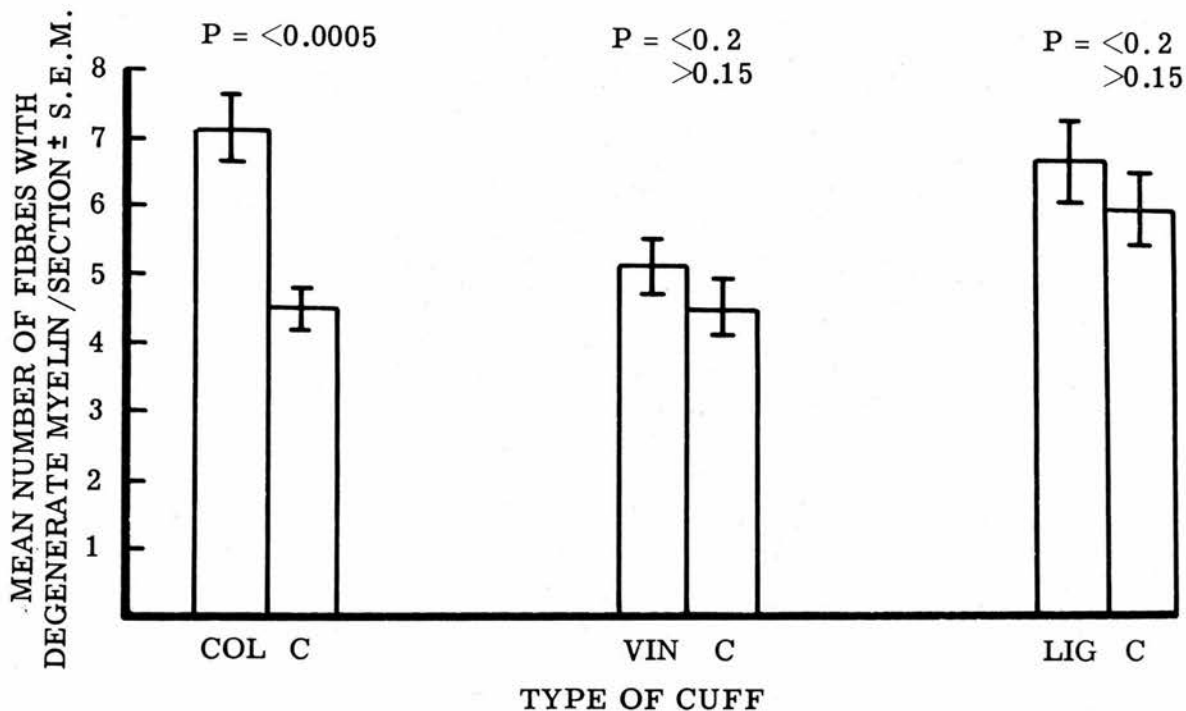


FIG. 54. Columns showing mean numbers of nerve fibres with degenerate myelin/section, with standard errors. The hypoglossal nerves received :

C = Control, plain silastic cuff.

COL = 0.1% colchicine cuff.

VIN = 0.1% vinblastine cuff.

LIG = 20% lignocaine cuff.

Note : colchicine causes a significant increase in myelin degeneration.

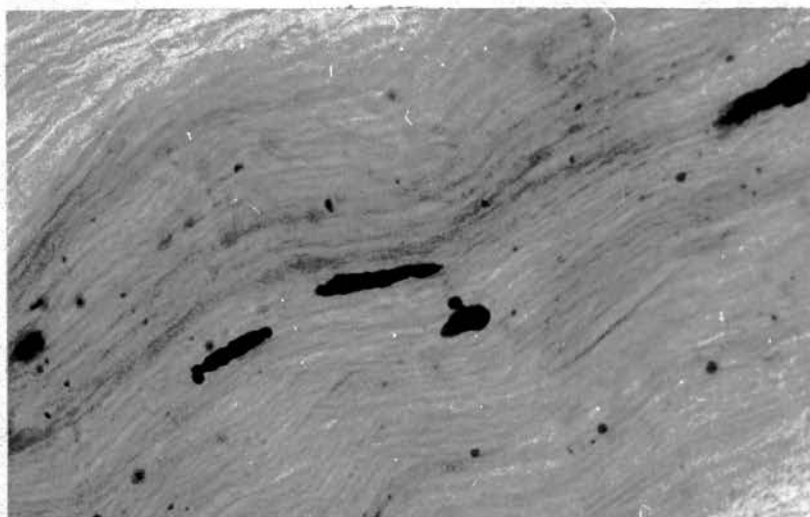


FIG. 55. Hypoglossal nerve treated with plain silastic cuff, stained for degenerate myelin.

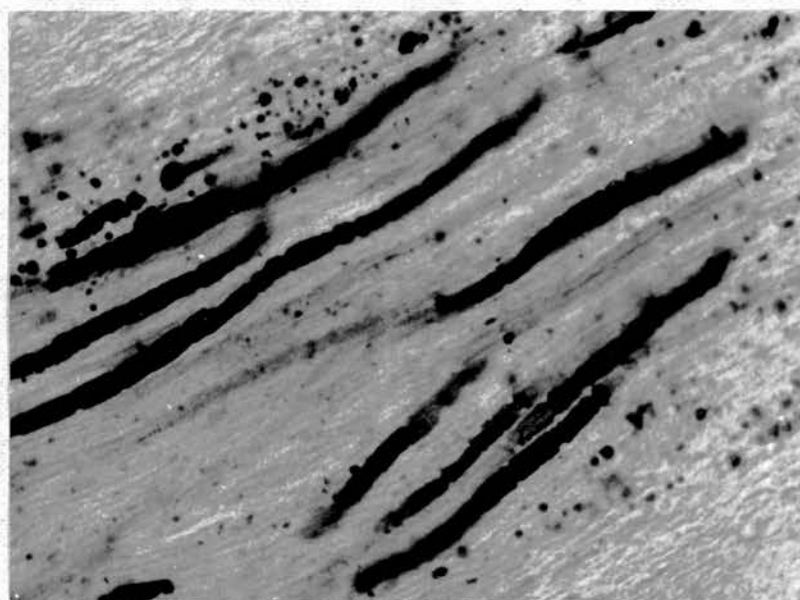


FIG. 56. Hypoglossal nerve treated with 0.1% colchicine cuff, stained for degenerate myelin. Note : increased numbers of darkly stained fibres with degenerate myelin (cf. Fig. 55.)

TABLE 10

Drug Group	Mean number of fibres with degenerate myelin/section				
	Left	S.E.M.	Right	S.E.M.	P
1 0.1% Colchicine	7.1	0.5	4.5	0.3	<0.0005
2 0.1% Vinblastine	5.1	0.4	4.5	0.4	<0.2 >0.15
3 20% Lignocaine	6.6	0.6	5.9	0.5	<0.2 >0.15

DISCUSSION

Horseradish peroxidase (HRP), a protein of molecular weight 40,000 daltons, reacts in the presence of hydrogen peroxide, with diaminobenzidine to form a brown product of unknown composition (197). Nerve cell bodies and axon terminals take up HRP from the extracellular space by pinocytosis (31-32). In nerve terminals, the protein is found inside membrane-bound vesicles of 43-50 nm diameter, which resemble synaptic vesicles (39). In more proximal axons, larger vesicles, sacs and multi-vesicular bodies are filled with HRP (39; 110). Nerve stimulation increases HRP uptake (30; 195), presumably by speeding up the turnover of synaptic vesicles. Peroxidase taken up by nerve terminals is actively transported to the parent neurone perikarya (39; 110-112). The role of microtubules in axonal transport has already

been discussed (Introduction and Section 5).

This series of experiments was carried out to determine the relationships between various kinds of axonal injury and the transport of exogenous protein from muscle to nerve cells. The results of part (a) indicate that there is no significant uptake of protein from muscle until the regenerating axons have made effective contacts with muscle. Motor end-plates would thus seem necessary for protein uptake. The increased number of neurones showing HRP uptake during the third week after axotomy has also been reported by other workers (110); it suggests that the turnover of terminal membrane may be increased during the early stages of reinnervation.

Nerves implanted into normally innervated sternomastoids did not show uptake of HRP injected into the muscle; but a small amount of uptake could be detected 11 days after division of the spinal accessory nerve (part (b)). Since nerves implanted into already innervated muscle rarely form end-plates (45-46) but do so if the muscle is denervated (42; 45-46; 48; 180) these results confirm the importance of effective nerve-muscle contact in the uptake of exogenous protein.

The presence of HRP-containing neurones on the right side after injections into the left sternomastoid (supplied by the left hypoglossal nerve) is surprising. A small proportion of hypoglossal neurones became chromatolytic after contralateral axotomy (personal observations); they presumably send their axons down the contralateral hypo-

glossal nerve. The relatively large number of contralateral neurones showing HRP uptake from sternomastoid might mean that long-term nerve transplantation induces more fibres to cross.

The results of part (c) indicate that axons regenerating within silastic chambers take up much less HRP than nerves with functional contacts on muscle (c.f. part (a)). Some possible reasons for this poor uptake have already been discussed (see section 6). In addition, the axons may have been damaged while being led into the chambers. The results suggest that muscle extracts failed to have significant effects (see section 6) on boutons because of poor axonal uptake from the tubes.

The experiments in part (d) confirm that the colchicine and vinblastine cuffs significantly inhibited axonal transport. These findings accord with the known properties of these drugs (section 5). More complete inhibition of axonal transport could probably have been achieved with larger cuffs, containing more drugs; but these have the dangers of direct mechanical and chemical damage to axons. These factors would only have confused the results, and so it seemed reasonable to accept partial inhibition of transport. Lignocaine cuffs caused some reduction in HRP transport, but this did not reach statistical significance. As discussed earlier (section 5), lignocaine is probably a weaker inhibitor of axonal transport than the other two drugs.

Staining for myelin degeneration was undertaken

because of the thinning of axons seen in silver stained sections of drug treated nerves (section 5). Only colchicine caused a significant increase in the number of fibres with myelin degeneration. The myelin may have degenerat@d because nutrients normally reaching the Schwann cells by axonal transport (108) had been held up. Alternatively, the fibres with myelin degeneration may have been injured by the cuffs directly. About 20-25% of the total fibres in the cuffed area showed myelin degeneration after colchicine. Since HRP transport was inhibited by more than 50%, colchicine cannot have acted purely by causing axonal death.

These results indicate that, while axon tips will take up extracellular protein to a limited extent, uptake is much greater if the nerves have functional nerve-muscle contacts. 0.1% colchicine and 0.1% vinblastine cuffs both significantly inhibit the transport of HRP to hypoglossal neurones; 20% lignocaine cuffs do not. But higher concentrations of lignocaine would probably also prevent axonal transport.

8. EFFECT OF SILASTIC CUFFS IMPREGNATED WITH COLCHICINE ON NERVE REGENERATION.

METHODS

Operations

20 rats were divided into two equal groups.

Group 1. The left hypoglossal nerve was crushed as it passed deep to the posterior belly of the digastric muscle. A silastic cuff containing 0.1% colchicine was applied to the crushed region, as in sections 5 and 7.

Group 2. The left hypoglossal nerve was crushed, as above, and a plain silastic cuff applied to the crushed region.

Electrophysiology

Between 5 and 26 days after operation, rats were anaesthetised with urethane and EMG measurements made. A bipolar needle electrode was used as before; it was inserted to a depth of 1.5 cm from the tongue tip into the corresponding side of the intrinsic muscle. The EMG spike voltage to maximal nerve stimuli was recorded as before (sections 5 and 7). The response on the left is expressed as a percentage of that on the right, and plotted against time after axotomy in FIG. 57.

RESULTS

The EMG response reappeared at the same time after axotomy in both control and drug treated rats. The course of regeneration was not clearly different in the two groups. (See FIG. 57).

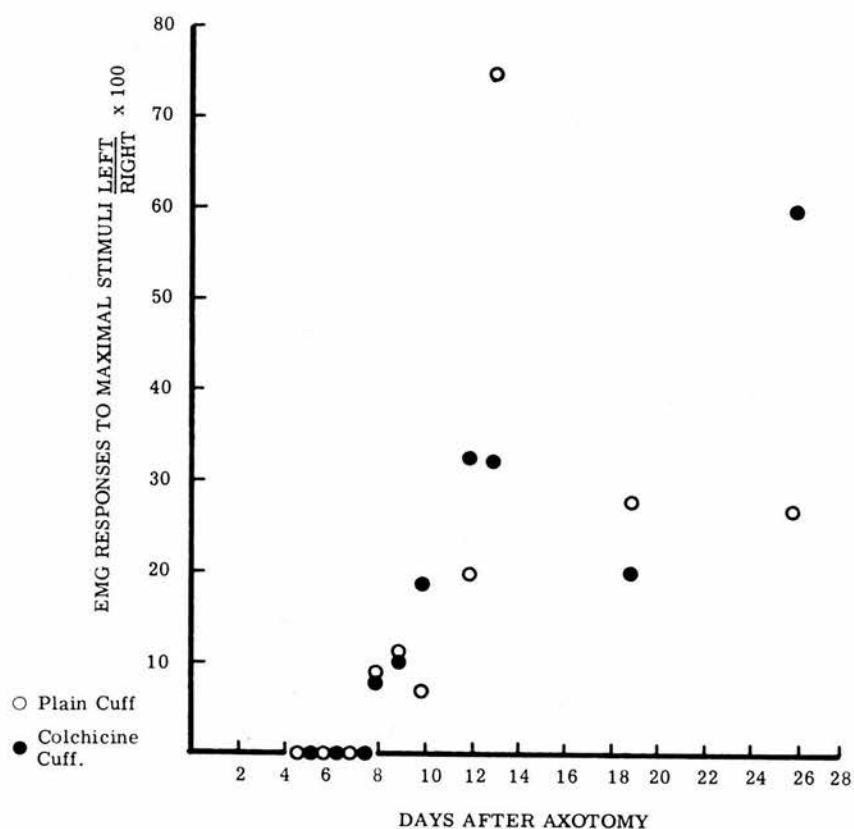


FIG. 57. Graph showing EMG responses recorded from the tongue, to maximal hypoglossal nerve stimuli. Percentages $\frac{\text{Left}}{\text{Right}} \times 100$ are plotted against days after left hypoglossal axotomy.

The crushed hypoglossal nerves received either a plain silastic cuff (open circles) or a cuff containing 0.1% colchicine (dark circles). Note : The course of nerve regeneration is not clearly different in the two groups.

DISCUSSION

Colchicine cuffs cause bouton loss when applied to intact nerves, and prevent bouton return when applied to regenerating transplanted nerves (section 5). Since the latter effect could be due to delay in axon growth, the present work was undertaken to see if similar cuffs would affect the regrowth of injured axons to muscle.

During nerve regeneration the distal axons are engulfed by Schwann cells (198-199) and the latter proliferate to fill up the endoneurial spaces in the distal stump (179). A Schwann cell scar grows out and bridges across the distal and proximal fragments (179; 200). Immediately after nerve injury, the proximal axons appear to retract about 2 mm into their stump (201-202); but soon they put out fine sprouts which move out into the Schwann cell scar and advance down the distal stump (179; 200).

In tissue culture, axons grow by extending thin tapering "microspikes" out into the medium. (88; 90). The rôle of actin-like microfilaments in the movements of "microspikes" has already been discussed (see Introduction). If proximal parts of growing axons are marked with carmine particles, they remain at a fairly constant distance from the neurone perikaryon (91). This suggests that nerves grow by adding new material to their ends rather than by moving the whole axon.

Axonal transport of proteins, glycoproteins and phospho-lipoproteins occurs in both directions (135), and

could be the source of new axon materials. The amount of protein transported up and down nerves increases after axotomy; and this appears to be independent of increased protein synthesis (113-114).

Since colchicine interferes with axonal transport, it might be expected to inhibit nerve regeneration. The extension of neurites from cultured neurones stops soon after colchicine is added to the medium; but the growth cone continues to put out "microspikes" for some time (98). The present results indicate that the silastic cuffs containing 10 μ gm of colchicine do not affect the rate of nerve regeneration. High concentrations of colchicine (300 μ gm injected into cat sciatic nerves) stop cut axons sprouting (192); but such a dose might interfere with more than just axonal transport.

Cuffs similar to the ones used in the present study inhibit retrograde axonal transport in some of the axons in the hypoglossal nerve (see section 7); whether they affect anterograde transport is unknown. The apparently normal rate of regeneration by colchicine-treated nerves could be due to three possibilities :

- (1) The cuffs do not inhibit anterograde axonal transport.
- (2) Nerve regeneration does not rely on axonal transport.
- (3) The fibres in the nerve which were not affected by colchicine would have regenerated at a normal rate; delayed regeneration in other fibres may have escaped detection, due to wide variations

between animals.

The last possibility seems the most likely one.

9. REFLEX RESPONSES FROM MUSCLES WITH NORMAL AND TRANSPLANTED NERVE SUPPLIES.

The experiments described here were undertaken to see if the contacts which formed on hypoglossal neurones with transplanted nerves were different from those on normal neurones.

METHODS

Operations

Six rats were studied. The left hypoglossal nerve was divided, and its central stump implanted into the ipsilateral sternomastoid muscle as in section 3 (a). Four months later, the left spinal accessory nerve was divided as it entered the superior end of the sternomastoid, and its central stump was buried in the neck muscles.

Electrophysiology

6-12 weeks after dividing the left spinal accessory nerve, rats were anaesthetised with urethane. EMG activity was recorded from the intrinsic tongue muscles and sternomastoids, using a bipolar needle electrode, as previously described (section 1). The electrode was moved about the muscles until activity from a small number of motor units could be distinguished on the oscilloscope or heard on the audio system. Representative responses were photographed from the oscilloscope screen.

Stimulation was entirely mechanical. Two types of stimuli were applied by squeezing either the lower lip or the ipsilateral forepaw with forceps. Responses from

the left sternomastoid muscle were recorded before and after division of the implanted hypoglossal nerve.

RESULTS

Results are shown in TABLE 11. In all six rats, motor units in the innervated (right) side of the tongue responded with a burst of rapid spike discharges when the lower lip was stimulated; but did not respond when either forepaw was squeezed.

Units in the normally innervated (right) sternomastoid muscle responded with bursts of spikes to stimulation of the forepaws; but their background discharge was inhibited when the lower lip was squeezed.

In four animals (Rats 1, 2, 3, and 6), units in the left sternomastoid (innervated by the left hypoglossal nerve) responded with spike activity to lip stimuli; but remained silent when the paws were squeezed. The response to lip stimulation was abolished after the transplanted hypoglossal nerve was cut.

In one of the other rats (Rat 5), the left sternomastoid did not respond to lip stimuli; but became active when the forepaws were squeezed. Division of the implanted hypoglossal nerve did not abolish this response; but dissection revealed a filament of the accessory nerve that had either regenerated or had not been cut at operation.

In the remaining rat (Rat 4), the left sternomastoid responded to both lip and forepaw stimuli; both responses were abolished by cutting the implanted hypoglossal nerve. However, the response to paw stimulation consisted only of a slight increase in the rate of background discharge.

TABLE 11

		Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
Site Stimulus	R. tongue lip	+++	+++	+++	+++	+++	+++
Site Stimulus	R. tongue forepaws	-	-	-	-	-	-
Site Stimulus	R. s/mastoid forepaws	++	++	+	+	+	+
Site Stimulus	R. s/mastoid lip	-	-	-	-	-	-
Site Stimulus	L. s/mastoid lip	+	++	++	++	-	+
Site Stimulus	L. s/mastoid forepaws	-	-	-	+	+	-
Site Stimulus	L. s/mastoid lip (L. XII) (divided)	-	-	-	-	-	-
Site Stimulus	L. s/mastoid forepaws (L. XII) (divided)	-	-	-	-	+	-
		EMG Responses					

Photographs of typical responses are shown in
FIGS. 58-64.

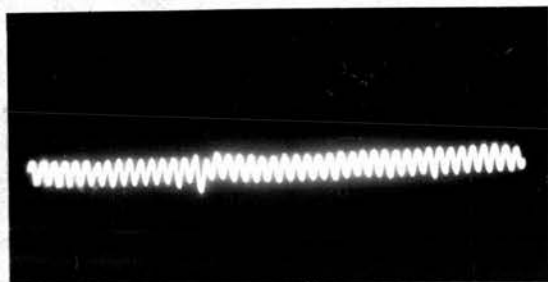


FIG. 58.

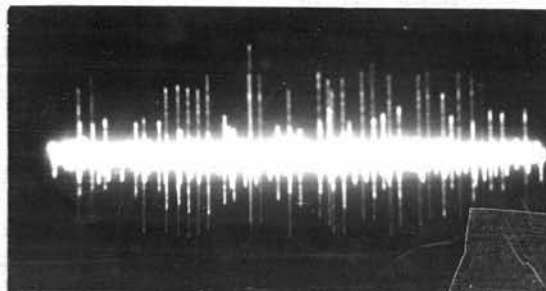


FIG. 59.

FIGS. 58 & 59. EMG records from the intrinsic tongue muscles, with no stimulation (Fig. 58) and during squeezing of the lower lip (Fig. 59).

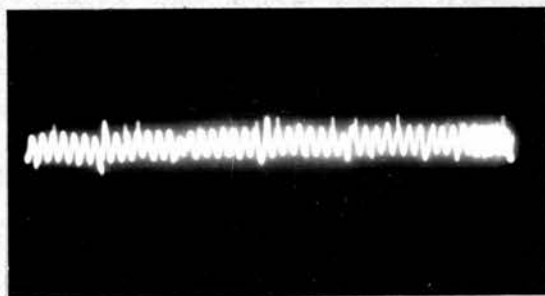


FIG. 60.

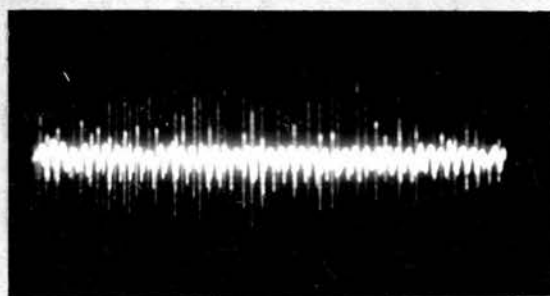


FIG. 61.

FIGS. 60 & 61. EMG from normally innervated right sternomastoid muscle, before (Fig. 60) and during squeezing of the right forepaw (Fig. 61).

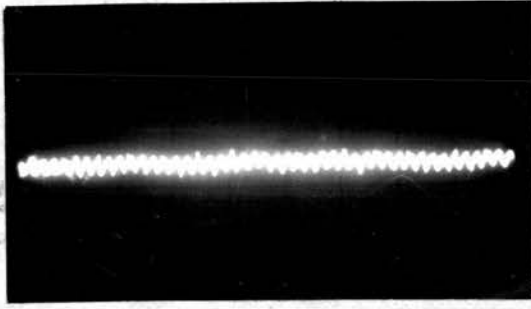


FIG. 62.

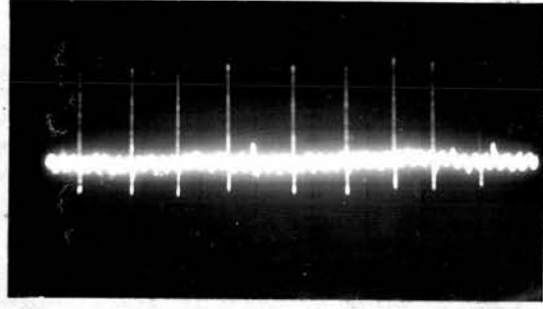


FIG. 63.

FIGS. 62 & 63. EMG records from left sternomastoid muscle innervated by a transplanted left hypoglossal nerve, before (Fig. 62) and during squeezing of the lower lip (Fig. 63).

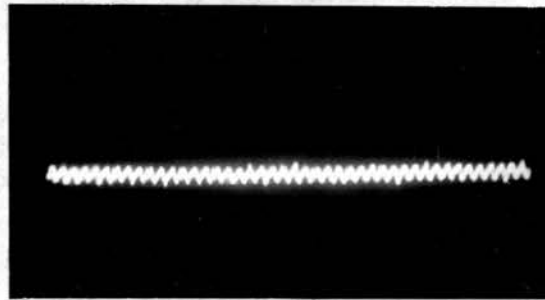


FIG. 64.

FIG. 64. EMG record from left sternomastoid muscle innervated by a transplanted left hypoglossal nerve, during squeezing of the left fore-paw.

DISCUSSION

The number of synaptic boutons on hypoglossal neurones falls after division of the hypoglossal nerve, and remains low if the nerve is implanted into the normally innervated sternomastoid muscle (section 3 (a)). Division of the ipsilateral spinal accessory nerve at this stage permits the hypoglossal nerve to innervate the sternomastoid (section 5 (d)), and is followed by a slow restoration in the number of boutons on hypoglossal neurones. The experiments described here were undertaken to see if the contacts which formed on hypoglossal neurones with transplanted nerves were different from those on normal neurones.

Reflex activation of normal hypoglossal neurones by stimulation of the lower lip is almost certainly relayed by interneurones in the descending nucleus of the trigeminal nerve. Similar responses to lingual nerve stimuli have already been discussed (section 4). Both types of stimulus cause a tongue-retraction reflex.

Reflex activation of the normal sternomastoid muscles evoked by forepaw stimulation is probably part of a polysynaptic flexor withdrawal reflex. The afferent fibres must be carried in the lower cervical roots. There was often prolonged after-discharge following a brief paw stimulus; and this was frequently inhibited by lip stimulation. In all the rats examined, normal sternomastoids did not respond to lip stimuli, and there was no tongue response when the paws were squeezed.

The results indicate that hypoglossal neurones, whose

axons have innervated sternomastoid muscle, retain their normal pattern of reflex activation. In only one case (Rat 4) did the transplanted nerve respond to paw stimulation. In this rat there was a background discharge in the sternomastoid; the frequency of this increased after both paw and lip stimulation. Division of the hypoglossal nerve abolished both responses. The latter case can be interpreted in two ways: either afferent fibres from the lower cervical roots have made their way to the hypoglossal neurones and formed "aberrant" contacts; or, and more likely, paw stimulation caused sufficient arousal of the reticular activating system that hypoglossal neurones were excited non-specifically.

The bulk of the evidence indicates that the boutons which form on hypoglossal neurones after nerve transplantation are of the same origin as those supplying normal neurones. Synaptic contacts from "aberrant" afferents appear to be either minimal or non-existent. This is supported by the absence of nerve growth cones in electron micrographs of hypoglossal nuclei during the phase of bouton restoration (203). The axons which form synapses on the regenerated neurones are probably already nearby.

In normal cats, only 6% of peroneus motor neurones are excited by stimulation of afferents in the nerve to lateral gastrocnemius. But, 21-38 weeks after the nerve to peroneus has been cross-united with the nerve to medial gastrocnemius, about 40% of the peroneus neurones are activated by stimulation of the nerve to lateral

gastrocnaemius (204-205). Cutting afferent fibres alone does not cause significant changes in the responses of motoneurones, after they have been re-innervated by collateral afferent sprouts (206). The acquisition of a new synergist thus appears to promote new and "aberrant" connections to form an axotomised neurones.

These latter experiments (204-206) involved two groups of neurones, anatomically closely related; both groups were axotomised simultaneously, and probably regenerated at similar rates; afferent fibres were also cut in both groups. In the present study, however, the hypoglossal and spinal accessory neurones are more widely separated; axotomy of their motor nerves was not synchronous; and sensory nerves were not injured. The two groups of neurones were at different phases of regeneration; and there would have been little time for afferents in the accessory nucleus to find their way to the hypoglossal nucleus before effective nerve-muscle contact was restored and boutons replaced.

It is therefore suggested that, for significant exchange of afferent connections to occur, the groups of neurones involved must be anatomically close; be axotomised and regenerate synchronously; and some afferent fibres be injured. Hypoglossal neurones innervating sternomastoid muscles do not fulfil these criteria sufficiently, and so no significant changes in synaptic connections take place.

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

The work presented here indicates that the number of synaptic boutons on the somata of rat hypoglossal neurones falls after motor nerve axotomy. Although this finding could be due to more boutons than usual being pulled off the altered surface of the injured neurones during shrinkage, comparable electron microscopic studies indicate that many boutons become separated from axotomised neurones by thin glial processes (170; 176). The changes in bouton numbers described in the present study are therefore likely to be real, rather than apparent.

The finding that only about half the total number of somatic boutons are removed after axotomy is interesting; perhaps an axon with several terminal branches withdraws some, but not all, of its contacts from the post-synaptic cell. Pre-synaptic terminals which have been removed from axotomised neurones lose their characteristic features, since the number of boutons in the neuropil decreases after axotomy (177).

The mechanism of bouton withdrawal is unclear: nerve terminals contain contractile proteins (133-134) which could be responsible for retracting the bouton. During bouton removal, the neuropil contains increased numbers of profiles with unusual inclusions (177). These inclusions are vacuoles, large clear vesicles, smooth endoplasmic reticulum, coated and dense-core vesicles, dense bodies and lamellated membranous bodies (autophagic

vacuoles). In addition, nerve terminals surrounding axotomised hypoglossal neurones take up increased amounts of protein from the extracellular space by pinocytosis (203). These findings suggest that retraction involves a process in which the bouton membrane is engulfed by its own pre-terminal axon.

It is not known how changes in the post-synaptic membrane influence the pre-synaptic terminals. Axotomised sympathetic neurones become progressively less responsive to applied acetyl choline (67-168). This suggests that the number of transmitter receptor sites might decrease, but could also indicate failure of coupling of receptors to ionophores. Regenerating optic axons fail to synapse with tectal neurones after acetyl choline receptor sites have been blocked with α -bungaro-toxin (78). Neurones may reject synapses by removing receptor sites from their surface. Other evidence for the importance of post-synaptic structures is seen in sub-synaptic cisterns which disappear after axotomy, but reappear before boutons are replaced (77). Thus synaptic sites might be determined by the location of specialised areas on the surface of the post-synaptic cell.

The relationship between a motor axon and its muscle governs the acceptance or rejection of synapses at the neurone perikaryon. Boutons are removed from the neurone if effective nerve-muscle contact is lost. They only return after functional neuromuscular junctions are reformed. When bidirectional axonal transport is blocked with colchicine or vinblastine, boutons are shed from the parent

neurones, even though effective nerve-muscle contact persists. It is therefore suggested that messenger materials derived from muscle are taken up into motor neurones at the neuromuscular junction, transported to the motor neurones by a mechanism involving microtubules, and control the properties of the neuronal surface by regulating the expression of genetic material. Proteins injected into muscle are certainly taken up into nerve terminals (29-32) and are transported to their cell bodies (39; 108; 110-112; 114). Furthermore, if labelled amino-acids are injected into muscle, activity appears in proximal axons in a form which is probably protein (136). Actinomycin-D, which binds to nuclear DNA capable of synthesising RNA, is bound in increased amounts by the nuclei of axotomised neurones (67). It is therefore suggested that molecules from muscle regulate gene expression in motor neurones.

The boutons which return to neurones that have regained effective contact with muscle probably come from the axons which formed the original synapses. The present study has shown that hypoglossal nerves transplanted to sternomastoid retain their original reflex patterns when boutons are restored. Further, it is unlikely that new axons grow into the injured nucleus, since growth cones are not detected while boutons are restored (177; 203). Lingual nerve division impedes the restoration of boutons, either because it decreases electrical activity in the afferent fibres, or because trans-synaptic degeneration (see Introduction) affects some of the terminals.

Regenerating neurones may receive unusual afferent connections under certain circumstances. If two adjacent groups of neurones are axotomised simultaneously, their motor nerves made to innervate the other's muscle, and if afferent fibres are cut at the same time, when a small proportion of "aberrant" reflex responses can be detected (204-206). Such an effect may relate more directly to the simultaneous occurrence of axotomy and degeneration of afferent fibres, rather than to innervation of an unusual muscle.

In conclusion, the present study indicates that the state of the terminal of a motor axon can determine whether boutons are retained, rejected or accepted by the cell body (and dendrites), but cannot regulate the type of synaptic connection formed in the adult rat. The work (Experimental Section 4) which demonstrates that the restoration of boutons is influenced by the sensory information they transmit is potentially an interesting bridge between morphogenesis, the response to injury, and excitation-metabolism coupling.

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APPENDIX

1. ELECTRON MICROGRAPHS OF Z10-STAINED TISSUE.

These electron micrographs were kindly prepared by Miss C.H.M. Ivens from tissue supplied by the author. The following procedure was used :

- (i) An adult rat was anaesthetised with ether and the vascular system perfused with 20 ml 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4), after a pre-wash of 20 ml normal saline.
- (ii) The medulla was removed (as in section 2) and left in the above fixative for 15 minutes.
- (iii) 0.4 mm transverse slices were cut through the hypoglossal area of the medulla on a McIlwain tissue chopper, and the slices impregnated in Z10 mixture (see section 2) overnight.
- (iv) The slices were dehydrated in graded ethanol solutions and small pieces bearing the hypoglossal neurones were embedded in araldite.
- (v) Ultrathin sections were cut and stained for 10 minutes in uranyl acetate; and 4 minutes in lead citrate.
- (vi) Sections were examined with an AEI EM 6B electron microscope.

Electron micrographs are shown in FIGS. 65-67.

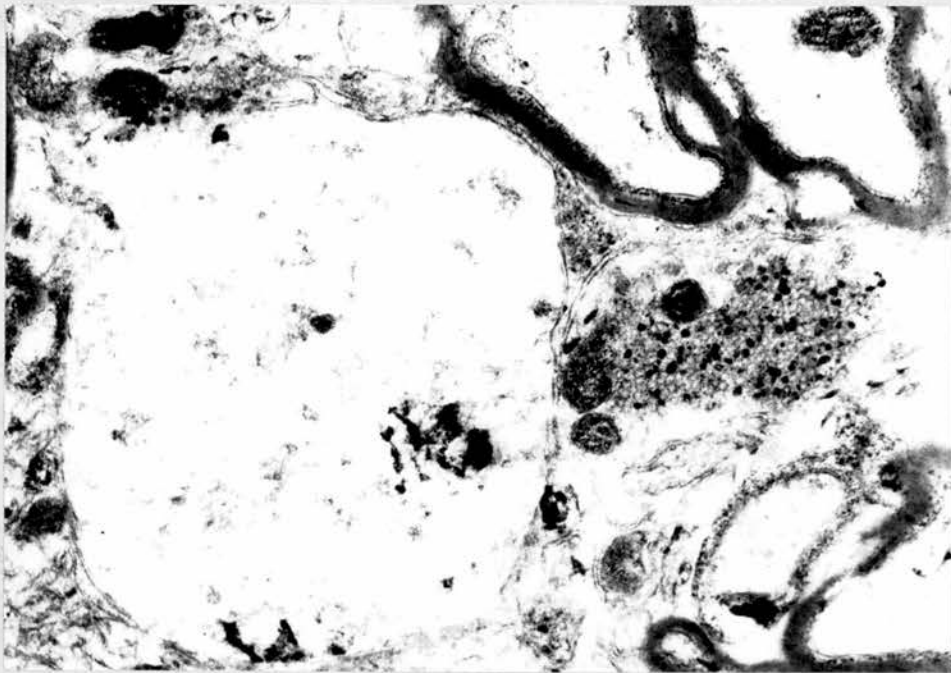


FIG. 65. Electron micrograph. Z10 preparation of hypoglossal nucleus X 15,000. An axo-dendritic synapse is seen, showing dense staining of synaptic vesicles and mitochondria.

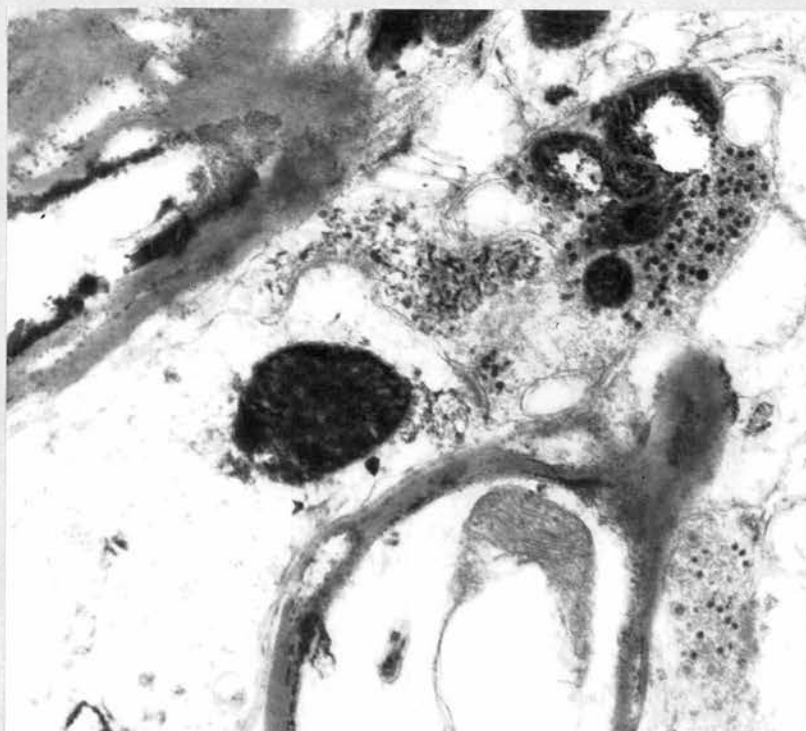


FIG. 66. Electron micrograph. Z10 preparation of hypoglossal nucleus X 20,000. Presynaptic boutons are seen, containing densely stained vesicles and mitochondria.

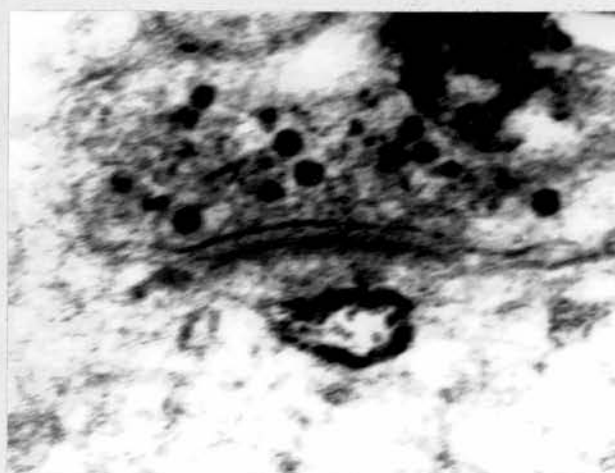


FIG. 67. Electron micrograph. Z10 preparation of hypoglossal nucleus X 60,000. A single synapse is seen; synaptic vesicles are densely stained on the presynaptic side.

2. PUBLICATIONS

- (i) [^]Role of Nerve-Muscle Contact in Maintaining Synaptic Connections.

Experimental Brain Research (1974) 20, 307-310.

- (ii) Effect of Sensory Nerve Division on the Afferent Synapses of Axotomised Motor Neurones.

Experimental Brain Research (1975) 22, 421-425.

Rôle of Nerve-Muscle Contact in Maintaining Synaptic Connections

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Summary. Presynaptic terminals on rat hypoglossal neurones have been stained by the Zinc Iodide-Osmium method, and counted using light microscopy. After cutting one hypoglossal nerve the number of somatic boutons on the parent cell bodies fell. During nerve regeneration, boutons were restored to the injured cells. The latency and rate of this restoration varied according to the type of nerve injury. Boutons were not restored until after effective nerve-muscle contact had been made again.

Key words: Synaptic connections — Nerve-muscle contact

Introduction

Synaptic boutons are shed from the surface of axotomised motor neurones (Blinzinger and Kreutzberg, 1968; Hamberger, Hansson and Sjöstrand, 1970), and are restored after the injured nerve regenerates (Sumner and Sutherland, 1973). Quantitative electron microscopy takes such a long time that it is difficult to analyse many cells in a changing situation. This study was undertaken to see if similar results could be obtained more quickly using light microscopy; they can.

Methods

Animals

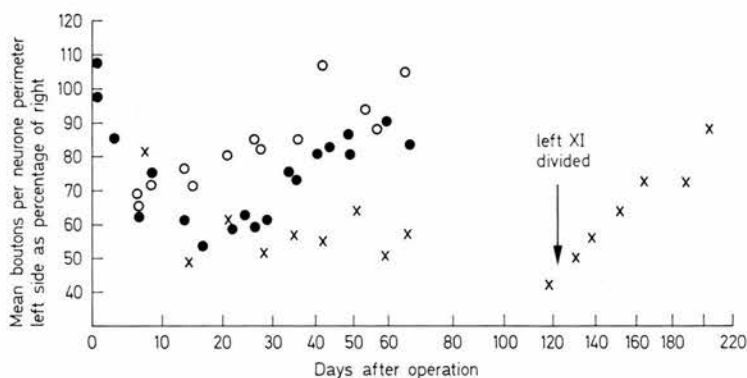
Observations were made upon male albino rats aged 3 months at the time of the first procedure, and between 3 and 10 months at time of death. They had free access to standard food pellets and water.

Operative Procedures

Five groups of rats were observed over a period of 14 months. Operations were carried out under ether anaesthesia. In the first group, the left hypoglossal nerve was divided as it passed deep to the posterior belly of the digastric muscle. In the second, the nerve was crushed at the same site. In the third, the left hypoglossal nerve was divided, and its central end implanted into the left sternomastoid muscle. The fourth group of rats was treated as in the third group, then, 123 days later, the left spinal accessory nerve was divided. The fifth group was normal rats.

Preparation of Tissue and Histological Technique

Between 3 and 120 days after operation, animals were anaesthetised with ether and exsanguinated. The medulla oblongata was rapidly removed, chilled, and chopped into transverse slices 0.4 mm thick, using a McIlwain tissue chopper. The separated slices were put into 2 ml of freshly prepared Zinc Iodide-Osmium (ZIO) mixture (Akert and Sandri, 1968) for 18—20



into sternomastoid, the number of boutons continued to fall; and by 120 days only 40% remained. Division of the left spinal accessory nerve at this stage was followed by a slow return of boutons.

Discussion

The ZIO method stains structures in nerve terminals which are probably vesicles; some of these vesicles are cholinergic (Parducz, Halasz and Joo, 1971; Akert and Sandri, 1968; Maillet, 1959).

If the results are compared with the recent electron microscopic study by Sumner and Sutherland (1973), good correlation is seen. A light microscopic method using silver impregnation of boutons has also given similar results (Cull, R. E., unpublished). The advantage of the ZIO method is speed.

Although neuronal swelling after axotomy could cause an apparent fall in density of boutons by increasing the surface area of the neurone, this cannot be held responsible for all the effects reported. First, cell swelling is over by the 30th day; but bouton changes persist, particularly in neurones whose axons are transplanted into sternomastoid. Second, electron micrographs of axotomised cells show glial processes separating nerve terminals from the neurones (Sumner and Sutherland, 1973).

The latency of the restoration of boutons appears to be directly related to the time at which the axons make effective contact with muscle. After crushing the hypoglossal nerve, regenerating fibres can be seen in the tongue 6 days later (Watson, W. E., personal communication). This precedes bouton restoration by about 7 days.

Transplanting the hypoglossal nerve into the normally innervated sternomastoid muscle permits neither regrowth back to the tongue nor innervation of the sternomastoid. Such nerve-muscle contact is not *effective* and is followed by persisting bouton loss on hypoglossal neurones. If the spinal accessory nerve is then divided, the transplanted hypoglossal nerve will innervate the sternomastoid, forming effective contact in about 6 days (Watson, 1970); restoration of boutons to the hypoglossal neurones follows this.

The form of the signal from muscle to nerve cell is unknown. It is not reliant on the passage of action potentials down the nerve, since prolonged local anaesthetic block of one hypoglossal nerve does not cause bouton changes (Cull, R. E., unpublished). Treatment of one hypoglossal nerve with colchicine leads to loss of somatic boutons; but this may not be due to impaired transport by microtubules, since axonal damage also occurs (Cull, R. E., unpublished).

This work provides further evidence for the importance of effective nerve-muscle contact in maintaining the central synaptic contacts of motor neurones.

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Effect of Sensory Nerve Division on the Afferent Synapses of Axotomised Motor Neurones

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Summary. Presynaptic terminals on rat hypoglossal neurones have been stained by the Zinc Iodide-Osmium method, and counted using light microscopy. Ipsilateral lingual nerve division did not alter bouton numbers on normal neurones. However, the restoration of bouton number after hypoglossal axotomy was inhibited significantly by ipsilateral lingual nerve division.

Key words: Synaptic connections — Sensory nerve division

Introduction

After one hypoglossal nerve has been crushed, the number of synaptic boutons on the parent neurone cell bodies decreases (Cull, 1974). This loss of boutons is greatest 7 days after axotomy; injured neurones then have about 65% the number of boutons of uninjured controls. After effective nerve-muscle contact has been restored, the number of boutons returns to normal; by 28 days after nerve crush, the cells have about 80% of their full complement of somatic boutons. The present study was undertaken to see if division of the ipsilateral lingual nerve would affect the boutons on normal and regenerating hypoglossal neurones.

Methods

Animals

Observations were made upon male albino rats aged 3 months at the time of the first procedure and 4 months at the time of death. They had free access to standard food pellets and water.

Operative Procedures

Three groups of rats were operated under ether anaesthesia.

Group A. Contained 9 rats. The left lingual nerve was divided at the point where it crossed the submandibular duct.

Group B. Contained 8 rats. The left lingual nerve was divided as above, but, in addition, the left hypoglossal nerve was crushed as it passed deep to the posterior belly of the digastric muscle.

Group C. Contained 8 rats. The left hypoglossal nerve was crushed as above, but the lingual nerves were not injured.

Preparation of Tissue and Histological Technique

Twenty-eight days after operation, animals were anaesthetised with ether and exsanguinated. The medulla oblongata was rapidly removed, chilled and chopped into transverse slices 0.4 mm thick, using a McIlwain tissue chopper. The separated slices were put into 2 ml of

freshly prepared Zinc Iodide-Osmium (ZIO) mixture (Akert and Sandri, 1968) for 24 hours at 4°C. Next day, the slices were washed in 0.2 M phosphate buffer (pH 7.4), dehydrated in graded alcohols, cleared in benzene and embedded in paraffin wax. 3 μ sections were cut and mounted on glass slides.

Microscopy and Counting

Sections were examined under transmitted light at $\times 400$ magnification. The number of ZIO granules in contact with the nerve cell body perimeter was counted (s. Fig. 1). In order to minimise differences in stain penetration, identical numbers of neurones were studied from both left and right hypoglossal nuclei in any one section. To exclude subjective bias, the identification code on each slide was covered with opaque tape, and a randomly allocated working code printed on the tape. Thus, the observer assessed each slide "blind". From each animal, 100 neurones were assessed; equal numbers coming from right and left hypoglossal nuclei.

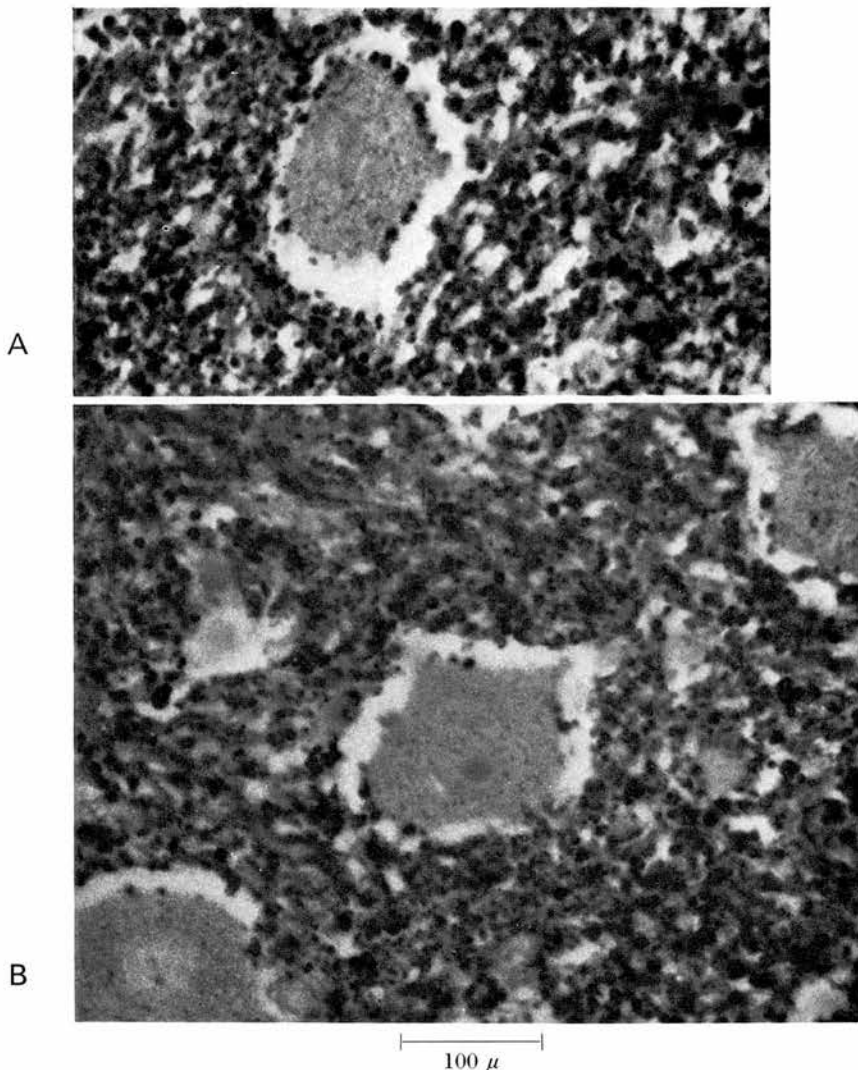


Fig. 1. Photomicrographs of hypoglossal motoneurons stained by ZIO: (A) Normal neuron. (B) Neurons 14 days after axotomy, showing loss of somatic boutons

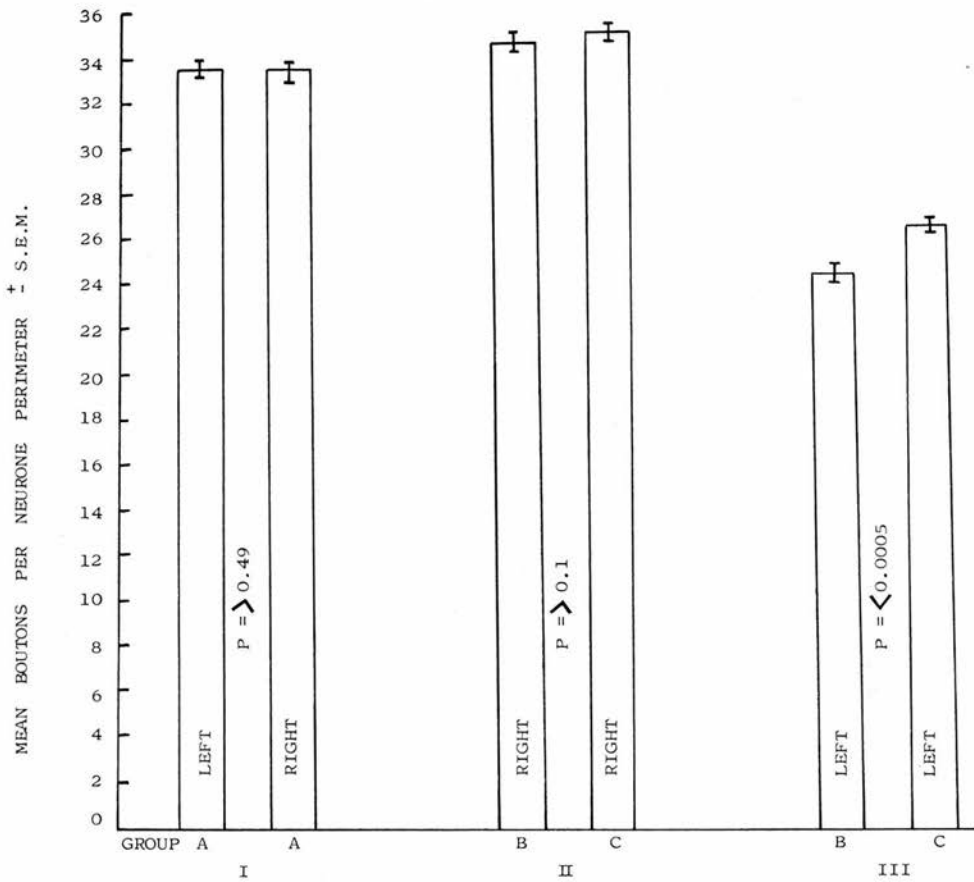


Fig. 2. Mean boutons per neurone perimeter together with standard errors. Significance of difference between each pair of values (P) calculated by Student's 't' test. Group A = Left lingual nerve divided. Group B = Left lingual nerve divided; left hypoglossal nerve crushed. Group C = Left hypoglossal nerve crushed. Note: Highly significant difference between left sides of Groups B and C ($P = < 0.0005$)

Expression of Results and Statistics

Group A. Bouton counts from 450 neurones (pooled data from 9 animals) in the left hypoglossal nucleus were compiled to give a mean value for boutons/neurone perimeter. The corresponding value for the right hypoglossal nucleus was calculated, and the two values compared by Student's 't' test.

Groups B and C. Bouton counts from 400 neurones (pooled data from 8 animals) in the left hypoglossal nucleus of Group B rats were compared by Student's 't' test with those derived from the left hypoglossal nucleus of Group C rats. Similarly, the right hypoglossal nucleus of Group B rats was compared with the right hypoglossal nucleus of Group C rats. All results are expressed in Fig. 2.

Results

The ZIO stained tissue was seen to be studded with black granules, distributed mainly in the neuropil and surrounding nerve cell bodies (Fig. 1). Division of the left lingual nerve alone (Group A; Fig. 2/I) did not cause any significant change in hypoglossal perisomatic boutons. However, left lingual nerve division together with left hypoglossal crush (Group B) inhibited significantly the restoration of bouton number to the injured neurones when compared with hypoglossal crush alone (Group C) ($P = <0.0005$; Fig. 2/III). The right hypoglossal neuronal boutons in Groups B and C did not differ significantly.

Discussion

The ZIO method offers a rapid way of detecting synaptic boutons, at least some of which are cholinergic (Párducz, Halász and Joó, 1971; Akert and Sandri, 1968; Maillet, 1959; Cull, 1974). Although ZIO has also been reported to stain mitochondria, smooth endoplasmic reticulum, Golgi apparatus and other intracellular organelles (Joó, Halász and Párducz, 1973), two points are worth considering. First, in the present study, structures stained at the nerve cell surface only were counted; thus excluding most of the material within the perikaryon. Counting was restricted to the perimeter of the neurone and any major dendrite continuous with this perimeter in the section (cf. Fig. 1). The counts therefore reflect mainly somatic boutons. Secondly, the timing and size of bouton changes after hypoglossal axotomy assessed by ZIO (Cull, 1974) correlate well with those obtained by a concurrently performed electronmicroscopic study (Sumner and Sutherland, 1973; Sumner, 1975). The ZIO method, using the counting techniques described, would therefore seem a valid way of estimating neuronal somatic boutons with the light microscope.

The results presented here indicate that, although ipsilateral lingual nerve division does not change the number of boutons on normal hypoglossal neurones, it does significantly inhibit the restoration of boutons to neurones whose axons are regaining contact with muscle.

Unilateral lingual nerve stimulation in the cat (Porter, 1965) and the rat (Duggan, Lodge and Biscoe, 1973) has both excitatory and inhibitory effects on hypoglossal neurones bilaterally. The ipsilateral excitatory response begins at 4–7 ms latency and is larger than the contralateral response which occurs some 1–2 ms later (Porter, 1967). A later, more variable, inhibitory effect frequently follows the initial excitatory response (Porter, 1967; Duggan, Lodge and Biscoe, 1973). It is probable that interneurones in the spinal nucleus of the trigeminal nerve relay this lingual-hypoglossal reflex (Porter, 1967).

Since the lingual nerve affects hypoglossal cells bilaterally and only via interneurones, it is not surprising that reducing impulse traffic down this pathway by lingual nerve section causes no significant bouton loss. What is important, is that during hypoglossal nerve regeneration, when synaptic contacts are in the process of reforming on the motor neurones, lingual nerve section significantly inhibits the restoration of bouton number. Whether the boutons concerned are those serving the lingual-hypoglossal reflex is unknown.

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